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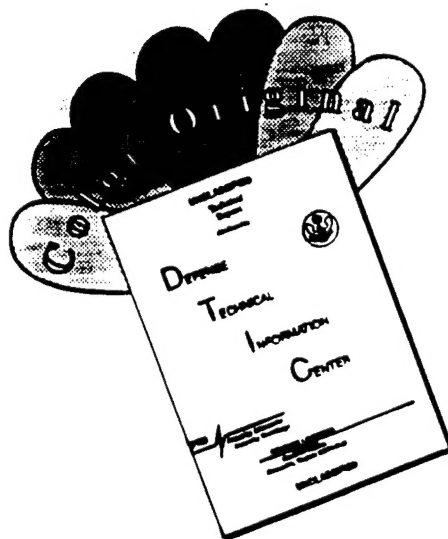
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1. Lisha Zhang, Ching-jer Chang, Sarah S. Bacus, and **Mien-Chie Hung**. Suppressed transformation and induced differentiation of HER-2/neu-overexpressing breast cancer cells by emodin. *Cancer Res.* 55: 3890-3896, 1995.
2. Nobutaka Kiyokawa, Duen-Hwa Yan, Mary Elizabeth Brown, and **Mien-Chie Hung**. Cell Cycle-dependent regulation of p185neu: a relationship between disruption of this regulation and transformation. *Proc. Natl. Acad. Sci. USA* 92: 1092-1096, 1995.
3. Susan J. Miller, and **Mien-Chie Hung**. Regulation of HER-2/neu gene expression. *Oncology Reports* 2: 497-503, 1995.
4. Youming Xie, Nobutaka Kiyokawa, Angabin Matin, and **Mien-Chie Hung** Signal transduction of HER-2/neu receptor tyrosine kinase. *The Cancer Bulletin* 47: 109-118, 1995

INTRODUCTION

A. Background

The data from the Surveillance, Epidemiology, and End Results (SEER) Program indicate breast cancer remains a major cause of death in this country (1). It was estimated that approximately 180,000 new cases of breast cancer will be diagnosed in the United States in 1995, and 46,000 women will die from this disease. Many oncogenes and tumor suppressor genes were shown to be involved in the development of breast cancer. The clinical value of each of these genes in prognostic and potential therapeutic applications has been studied to some extent (2). Yet, none of these molecular markers alone was found to be a better prognosis factor than the prognosis factors currently used for breast cancer patients (e.g. number of metastatic lymph nodes). **Identification of a set of related genes that are involved in breast cancer may be critical to develop a better molecular prognostic strategy. Studies on the role of a set of related genes and their interrelationship in breast cancer may also provide a more productive avenue to understand the basic biology of breast cancer cells.** Loss of estrogen receptor (ER) expression or function has been known for a long time to be associated with poor prognosis for breast cancer patients. The *HER-2/neu* oncogene encodes a growth factor receptor-like molecule and overexpression of *HER-2/neu* is also reported to correlate positively with poor survival for breast cancer patients. Some studies, including our own, demonstrate that estrogen-stimulated ER can repress *HER-2/neu* overexpression, suggesting a possible causal relationship between *HER-2/neu* overexpression and non-function of ER in breast cancer cells. We have also recently found that the tumor suppressor gene, Rb, can repress *HER-2/neu* expression. These studies raised an interesting possibility that ER and Rb may suppress the effects on the malignant transformation phenotype induced by *HER-2/neu* overexpression. The Rb gene is known to be inactivated in approximately 20% of breast tumors. It is not yet clear whether Rb might have prognosis value in breast cancer. The ligand (named Heregulin or NDF) for the *HER-2/neu* encoded receptor has recently been isolated and its cDNA cloned. In view of current knowledge about autocrine and paracrine interactions between a growth factor(s) and its cognate receptor(s), it is likely that Heregulin may also be involved in the development of breast cancer.

A-1. The *HER-2/neu* Proto-oncogene Encoding an Epidermal Growth Factor (EGF) Receptor-related Protein is a Potent Transforming Oncogene

The rat *neu* oncogene was a transforming gene originally isolated from rat neuroblastoma and later its cellular counterpart, the normal *neu* gene, was also isolated from rat and human libraries (3-6). Detailed structural and functional analysis of the transforming rat *neu* and the normal *neu* genes (*neu* proto-oncogene) indicates that a single point mutation in the transmembrane region is responsible for the conversion of the normal *neu* gene into a transforming *neu* oncogene (7). The human homologue (*HER-2* or *c-erbB-2*) of rat *neu* oncogene was isolated based on its homology to chicken *v-erbB* gene (*HER-2* represents Human EGF Receptor-2 and *c-erbB-2* means the second gene homologous to *v-erbB*; EGF receptor gene was the first). It is now known that *neu*, *HER-2*, and *c-erbB2* are the same gene. We will use *HER-2/neu* to represent this gene since *HER-2/neu* seems to be the most frequently used in the literature. Structural comparison between the *HER-2/neu*-encoded p185 protein and EGF receptor revealed significant sequence homology and identical gross structural organization including ligand-binding, transmembrane, and tyrosine-kinase domains between these two proteins (5-8). Both EGF receptor and p185 proteins can form either homodimers or heterodimers (9-11). The homodimer is believed to be an active receptor form for ligand binding. The function of heterodimer is not yet very clear. The ligand for the *HER-2/neu*-encoded p185 has recently been purified and its cDNA cloned (12-17). Recently, we and others have independently developed different techniques to search for possible mutation(s) in the transmembrane region of the human *HER-2/neu* gene (18, 19). So far, no mutation has been found in human tumor DNA. A possible interpretation for this discrepancy is that double mutations (from Val, GTT to Glu, GAA or GAG) are required for generating the same amino acid change in a human gene but only one single point mutation (from Val, GTG to Glu, GAG) is sufficient to generate the activation mutation in the rat *neu* gene. The mutated p185 protein has been shown to enhance tyrosine kinase activity (20, 21), which mimics the enhanced tyrosine kinase activity of p185 by overexpression of the normal *HER-2/neu* gene. This notion is further supported by the fact that higher levels of normal *HER-2/neu* are required for transformation of rodent fibroblast cell lines (22, 23), yet a low level of mutation-activated *HER-2/neu* oncogene expression is sufficient to transform the

same fibroblast cells (24). Therefore, the mutation-activated *HER-2/neu* oncogene serves as an excellent model gene to study the biological effects of overexpressed normal *HER-2/neu* gene.

A-2. Amplification/Overexpression of the Human *HER-2/neu* Gene is Frequently Found in Human Cancers including Breast Cancer.

Unlike the rat *neu* oncogene that is activated by a single point mutation, the human *HER-2/neu* gene is activated by overexpression in human cancers. Amplification/overexpression of the *HER-2/neu* gene was first found in approximately 30% of human breast cancers (25-30) and later in many other human cancers (31-36). In the case of breast, ovarian, lung and gastric cancers, several reports further indicated that *HER-2/neu* overexpression correlates with a poor survival rate (30-36), suggesting that *HER-2/neu* overexpression may be used as a prognosis factor (30-32, 25, 27). However, some studies disagree that *HER-2/neu* overexpression can be a poor prognostic factor in breast cancer (37-39). Although the discrepancy could be caused by reasons such as patient population and treatment differences, the methods and reagents used for detection of *HER-2/neu* overexpression and the way tumor specimens were collected, other more significant reasons may also contribute to this discrepancy. Considering the fact that the development of breast cancer requires multi-step activations, it is possible that *HER-2/neu* overexpression alone may not be an ideal prognosis factor for breast cancer patients. Combined data from the expression of multiple oncogenes and tumor suppressor genes involved in breast cancer may provide a more accurate prognosis. Both ER and Rb are shown to be involved in breast cancer and our studies also indicate that they can regulate *HER-2/neu* expression (see below). In addition, Heregulin, a ligand for the *HER-2/neu* receptor, which has recently been isolated and cloned may also contribute to the development of breast cancer thorough interaction with *HER-2/neu* receptor (see below). The current proposal will focus on interactions between *HER-2/neu* and ER, Rb or Heregulin in breast cancer and their effects on basic biology of breast cancer cells.

A-3. Identification of Multiple Ligands of *HER-2/neu* Receptor and Cloning of the Heregulin cDNA

It has been known since 1985 that the *HER-2/neu* encoded p185 protein has all the characteristics of a growth factor receptor. However, several ligands that binds to this receptor were only recently identified and one of them cloned (12-17). The human version of the ligand (named Heregulin) was detected from conditioned medium of MDA-MB-231 human breast carcinoma cells as a 45 kDa protein which interacts specifically with *HER-2/neu* encoded p185, stimulates breast cancer cell proliferation in culture and increases tyrosine phosphorylation of p185 (12). The rat version of the ligand was purified and cloned from the medium of *ras*-transformed rat 1-EJ cells with similar size as Heregulin (44 kDa) and similar biochemical properties, except that it inhibits rather than stimulates breast cancer cell proliferation in culture and induced differentiation of AU-565 breast cancer cells. Therefore, it was named *neu* differentiation factor (NDF) (13, 14). It is not yet clear what causes the differential response of human ligand, Heregulin and rat ligand, NDF to human breast cancer cells. In addition, a few other molecules have been reported to behave like ligands for p185 (12-17). It is likely that similar to the EGF receptor, multiple forms of ligands may exist for the *HER-2/neu*-encoded p185. Since Heregulin is the best characterized ligand, and the only ligand to which the detecting reagents such as cDNA clone and antibody are available, we will focus only on Heregulin in this proposal.

A-4. Non-function of ER May Contribute to *HER-2/neu* Overexpression in Some Breast Cancer

Steroid hormones play an essential part in regulating the growth of both normal and neoplastic breast cells. Specifically, estrogen has a marked effect on the proliferation of breast cells *in vivo* and *in vitro*. Although the mechanisms by which estradiol (E2) induces proliferation in estrogen receptor (ER) positive breast cells are incompletely defined, modulation in expression of certain growth related cellular proto-oncogenes by estradiol stimulated estrogen receptor (E2/ER) has been well described using cell lines established from human breast tumors. The function of ER in breast cancer is unclear; however, the significant correlation between loss of functional estrogen receptor and poor patient prognosis is very well-described (50,51). Evidence that ER may play a role in the regulation of *HER-2/neu* expression comes from several diverse observations. Several studies of human breast tumor tissue specimens have shown an inverse relationship between ER and *HER-2/neu* expression (52-55). Furthermore, during development of rat mammary glands, expression of *HER-2/neu* is inversely related to ER status (56). Those breast cancer cell lines with the highest levels of *HER-2/neu* overexpression

are generally ER negative or have very low levels of estrogen receptor. We and others have recently shown that ER through estrogen stimulation can negatively regulate the expression of *HER-2/neu* in ER positive but not ER negative breast cancer cell lines (57-59). We further demonstrated that the ER-mediated *HER-2/neu* repression can occur at the transcriptional level (59). The result suggests that *HER-2/neu* overexpression may be caused by inactivation of ER in some breast tumors. It also raises an interesting possibility that expression of ER in the ER negative and *HER-2/neu*-overexpressing breast cancer cells may suppress malignant transformation induced by *HER-2/neu* overexpression. If this indeed is correct, it may provide an interpretation for a well-known clinical phenomenon, namely, some ER positive breast cancer patients do not respond to hormone treatment such as Tamoxifen (TAM), an estrogen antagonist. **Our purpose is that ER positive breast tumors with *HER-2/neu* overexpression may not respond to TAM treatment since TAM competes with estrogen to interact with ER, and in one way blocks the stimulating effect of tumor cell growth (Estrogen can stimulate growth of ER positive cells), but in another way enhances *HER-2/neu* overexpression, which enables the tumor cells to become more malignant. The purpose 3 will examine whether ER can suppress malignant transformation of *HER-2/neu*-overexpressing breast cancer cells through E2/ER-mediated *HER-2/neu* repression, and if it is correct, also test the purpose mentioned above.**

A-5. The Tumor Suppressor Gene, Rb, May Suppress Tumorigenicity of Human Breast Cancer Cells with *HER-2/neu* Overexpression.

The retinoblastoma susceptibility gene (Rb) is a well-characterized tumor suppressor gene (60). The existence of this gene was initially predicted based upon genetic predispositions to certain pediatric malignancies (61). Tumor formation or transformation occurs when these genes are inactivated, suggesting that their normal function is to limit cellular proliferation. Inactivation or deletion of Rb has been found in a variety of human cancers including breast cancer (60). Using retroviral-mediated gene transfer, it has been shown that the Rb gene can suppress tumor formation of retinoblastoma, osteosarcoma, and breast carcinoma in which the endogenous Rb gene is inactivated (62-64).

The Rb gene encodes a 105kDa protein (RB) and is known to form a protein complex with adenovirus E1A protein as well as large T (LT) antigen of SV40 virus and E7 protein of papilloma virus (65-67). It is believed that the DNA virus-associated proteins such as E1A, LT and E7 may inactivate the RB function through RB-E1A (or LT, E7) complex. Biochemically, RB can function as a transcriptional factor that can regulate transcription of cellular genes including *c-myc*, *TGF- β* , *c-fos* and *HER-2/neu* (68-71). More recently, RB has been shown to form a protein complex with a DNA-binding protein E2F and may, therefore, act as a transcriptional factor by complexing with other factors (72, 73). We have previously demonstrated that E1A gene products inhibit *HER-2/neu* expression in both rodent and human breast cancer cells (74-76). The results allow us to further discover that E1A gene functions as a tumor suppressor gene for the *HER-2/neu*-overexpressing tumor cells through transcriptional repression of *HER-2/neu* gene. Since E1A and RB proteins can form E1A-RB protein complexes and it is believed that E1A proteins may inactivate RB function through E1A-RB complexes, our results which clearly demonstrated that E1A gene is a tumor suppressor gene for the *HER-2/neu* transformed cells seemed to be surprising at the beginning. However, it has recently been shown that E1A can stabilize the tumor suppressor, p53, and induce cell program death (Apoptosis) (77-80). This property strongly supports tumor suppressor function of E1A. We have now shown that RB alone can also inhibit *HER-2/neu* transformation through transcriptional repression (71). The effect of RB-E1A complexes on *HER-2/neu* expression is very complex as RB can enhance E1A-mediated *HER-2/neu* repression in some cell types but not in other cell types (our unpublished observation). Our preliminary results suggest an interesting phenomenon: that E1A and RB may cooperate to enhance a biological activity in one cell type while antagonizing each other to suppress a biological function in another cell type. This complex issue will not be addressed in the current proposal. Instead, we will focus on the RB effect on *HER-2/neu*-overexpressing breast cancer cells. If RB can also suppress malignant transformation of *HER-2/neu*-overexpressing cancer cells, the tumor suppressor function of the Rb gene will not be limited only to the Rb-defective tumors and may extend to the tumors with *HER-2/neu* overexpression. This will become an important issue when the gene therapy technique is developed well enough to allow delivery of the Rb gene into patient's cancer cells.

B. THE PURPOSES

The major purposes are:

B-1. Systematic studies on the expression of *HER-2/neu*, Heregulin, ER and Rb in breast tumor specimens and correlation of the expression with tumor stages and patient survival.

Our purpose is that the combination of *HER-2/neu*, Heregulin, ER and Rb may be a better prognosis factor than each of these molecules individually. Therefore, expression of *HER-2/neu*, Heregulin, ER, and Rb in the same breast tumor specimens will be examined by immuno-histochemical staining, *in situ* hybridization, and western and northern blots. The relationship between expression of these molecules, tumor grades and patients' survival will be evaluated.

B-2. Potential paracrine and autocrine interactions between *HER-2/neu* and Heregulin in breast cancer cells.

Potential paracrine and autocrine loops between *HER-2/neu* receptor and Heregulin ligand will be tested by using expression vectors and model cell lines. Effects of Heregulin on transformation phenotypes of breast cancer cells will be examined by growth properties, soft agar colonization assay, subcutaneous (s.c.) tumorigenicity and intraperitoneal (i.p.) survival assays.

B-3. Effects of ER on malignant transformation phenotypes of *HER-2/neu*-overexpressing breast cancer cells.

Since we have found that estrogen-stimulated ER can repress *HER-2/neu* gene expression, ER expression vectors will be used to modulate *HER-2/neu* expression in *HER-2/neu* overexpressing breast cancer cells. The effect of ER on transformation phenotypes of *HER-2/neu* overexpressing breast cancer cells will be examined. The potential effects of Tamoxifen, an estrogen antagonist, will also be tested in this system.

B-4. Effects of Rb on malignant transformation phenotypes of *HER-2/neu*-overexpressing breast cancer cells.

The Rb-expression vectors will be introduced into the *HER-2/neu* overexpressing breast cancer cells. The effects of Rb on *HER-2/neu* expression and transformation phenotypes will be analyzed. Potential relationship among Rb, ER and *HER-2/neu* will also be examined.

BODY:1. Systematic studies on the expression of HER-2/neu, Heregulin, ER and Rb.

Heregulin was originally thought to be a ligand for HER-2/neu (12-14). However, more recent data indicate that Heregulin is a ligand for HER-3 and HER-4 (also known as *c-erbB3* and *c-erbB4*) that are two recently identified EGF receptor family genes. Since HER-3 and HER-4 can form heterodimers with HER-2/neu and interactions between Heregulin and HER-2/neu is most likely through HER-3:HER-2/neu dimer or HER-4:HER-2/neu dimer (81-82), it is possible that HER-3 and HER-4 may also contribute to breast cancer. We will, therefore, include HER-3 and HER-4 in our expression study in breast tumor tissues.

We have used specific antibodies for HER-2/neu, HER-3, HER-4, Rb and Heregulin to stain the archival paraffin-embedded sections. Up to now, 120 tumor sections have been completed for HER-2/neu and HER-3. One set of representative data was shown in Fig. 1 (HER-2/neu) and Fig. 2 (HER-3). In both cases, we can identify tumors with high, intermediate, low and negative expression. The relationship between the expression level with ER status (available from medical records) tumor grades and patient survival is currently under investigation. We will continue to determine HER-4 and Heregulin expression levels in these tumor sections and analyze their relationship with tumor grades and patient survival.

Involvement of Rb in human cancer is primarily regulated by the non-function of Rb protein that could be derived from deletion or mutation of the Rb gene. In some cases, the aberrant Rb protein can still be expressed in tumors and therefore scored as "positive" by immunohistochemical staining. Although western blotting analysis may detect aberrant form of Rb protein, it may not be able to distinguish difference between single-point-mutated Rb and wild-type Rb protein. Therefore, immunohistochemical staining for Rb protein will not be a very informative strategy to detect inactivation of Rb in tumor tissues (This was also discussed in the original grant proposal.). As mentioned earlier, the newly identified molecules, HER-3 and HER-4, like HER-2/neu, belong to the EGF receptor family and interactions between Heregulin and HER-2/neu require existence of HER-3 or HER-4 (most likely through heterodimer between HER-3 and HER-2/neu or HER-4 and HER-2/neu). Overexpression of HER-3 and HER-4 can be easily identified by immunohistochemical staining (e.g. Please see Fig. 2). We feel it will be more informative to analyze HER-3 and HER-4 expression than to analyze Rb expression, since Rb expression detected by immunohistochemical staining does not really reflect its contribution to tumor development (as discussed earlier). We would, therefore, like to focus on HER-2/neu, HER-3, HER-4, Heregulin and ER in the expression studies.

2. Potential Paracrine and Autocrine Interactions between HER-2/neu and Heregulin in Breast Cancer Cells.

We have completed the construction of Heregulin-expression vectors and anti-sense Heregulin plasmids. However, because of the recent data indicating that Heregulin binds to HER-3 or HER-4 directly and does not bind to HER-2/neu (81-82) and the interaction between Heregulin and HER-2/neu is most likely through HER3:HER-2/neu or HER4:HER-2/neu dimers, it becomes critical to know the status of HER-2/neu, HER-3 and HER-4 in breast cancer cells in order to study potential paracrine and autocrine interactions for HER-2/neu and Heregulin. We have now performed western blotting analysis of a panel of breast cancer cell lines for their HER-2/neu, HER-3 and HER-4 expression levels. We have identified several breast cancer cell lines that express HER-3 and also overexpress HER-2/neu. These include MDA-MB-453, MDA-MB-361, SKBr3. One representative western blotting data is shown in Fig. 3 that clearly indicate HER-3 expression in MDA-MB-453, MDA-MB-231 and MDA-MB-435 breast cancer cell lines, but not in BT-20 cell line. MDA-MB-231 and MDA-MB-435 are known to express only basal level of HER-2/neu. To establish stable transfectants, Heregulin-expression vector will be transfected into HER-3 positive and HER-2/neu-overexpressing cells and anti-sense construct into Heregulin-expressing cells such as MDA-MB-231.

3. Effects of ER on Malignant Transformation Phenotypes of HER-2/*neu*-overexpressing Breast Cancer Cells.

Using our ER-transfectants (59), we have shown that estrogen indeed suppresses transformation phenotype of ER⁺, HER-2/*neu* breast cancer cells as predicted in our original proposal. We have started to work on a manuscript to report this study. The results are described as follows:

*The presence of E2/ER affects the ability of HER-2/*neu* to transform NIH/3T3 cells.* To test the hypothesis that regulation of HER-2/*neu* expression by E2/ER can prevent cellular transformation by HER-2/*neu*, a focus formation system was utilized. NIH/3T3 cells were transfected with DNA encoding transforming oncogene HER-2/*neu*. In each plate, only a limited percentage of these cells received and expressed the oncogene. The cells that were transformed by the oncogene piled up as they proliferated to form foci of transformed cells in a background of non-transformed NIH/3T3 cells. These foci were easily visualized and counted after staining. The number of foci formed is a function of the efficiency of gene transfer as well as the efficiency of expression of the oncogene-encoded product. In later experiments, the differences in gene transfer and maintenance were eliminated by comparing only plates of cells from a single transfection. This was achieved by splitting a single transfected plate into four identical plates for subsequent treatment with E2 (see legend Table I).

NIH/3T3 cells were transfected with the c-nu-104 expression construct, which contains the transforming rat *neu** (activated *neu*) oncogene under the control of its endogenous promoter [4]. Table I shows that the presence of E2 and ER dramatically reduced the ability of c-nu-104 to induce focus formation in NIH/3T3 cells. This result is consistent with our previous data that E2/ER can inhibit HER-2/*neu* expression and suggests that the decreased HER-2/*neu* expression results in reduced transformation phenotype.

In order to assess whether inhibition of NIH/3T3 transformation by E2/ER is a generalized phenomenon, these experiments were repeated using another transforming oncogene. The pT24-C3 construct contains a 6.4 kb portion of the human homolog to the H-*ras* oncogene. The gene is under control of its own promoter in this construct and has been shown to be a potent transforming oncogene in focus formation assays. When this construct was co-transfected with plasmid expressing estrogen receptor into NIH/3T3 cells, no difference in focus formation was seen between cells grown in the presence of E2 and those grown in the absence of E2 (Fig. 4). Therefore, the inhibition of HER-2/*neu* mediated transformation of NIH/3T3 cells by E2/ER is oncogene specific, in that E2/ER does not alter transformation by *ras*.

Estradiol inhibits the ability of BT-474 (i/ER) sublines to grow in soft agar. The previous findings suggest that, by repressing HER-2/*neu* expression, E2 and ER may play a role in regulating the ability of HER-2/*neu* to act as a transforming oncogene. To test this possibility in human breast cancer cell lines, the BT-474 breast cancer cell line, which is ER negative and HER-2/*neu*-overexpressing, and its ER transfectants (ER positive) were chosen for these experiments. The formation of ER-expressing subclones of the BT-474 human breast cancer cell line was previously described [59]. When ER⁺ (i/ER) and ER⁻ (i/neo) subclones were tested for their ability to grow in an anchorage-independent manner, we found that the ability of the (i/ER) subclones to form colonies in soft agar was significantly reduced in the presence of E2 (Figures 5 and 6). E2 had no effect on the ability of the ER⁻ (i/neo) subclone to form colonies in these experiments, which agrees with the idea that E2 alone cannot repress the expression of HER-2/*neu* in these cells [59]. These results clearly indicate that E2 can suppress transformation of ER⁺, HER-2/*neu*-overexpressing cancer cells but has no effect on ER⁻, HER-2/*neu*-overexpressing cells. This suppression is most likely caused by E2/ER-mediated HER-2/*neu* repression.

Estradiol stimulates the ability of BT-474 (i/ER) sublines to grow in monolayer culture. The results presented above agrees with data showing a correlation between increased HER-2/*neu* expression and cellular transformation (in this case, measured by anchorage-independent growth). The addition of E2/ER to these cells, both suppress HER-2/*neu* expression and lead to a reduction in their anchorage-independent growth. It is important to note that in other ER⁺ breast cancer cell lines (e.g., MCF-7 and ZR-75-1), E2 stimulates growth in monolayer cultures. Since growth in soft agar and in monolayer culture is not necessarily correlated, growth curves were constructed to assess the effects of E2 and ER on the growth of the BT-474 subclones in monolayer culture.

Table II shows the results from these experiments. Growth of the (i/ER) subclones was significantly enhanced in the presence of E2; whereas E2 had no effect on the growth of the ER⁻ control subclone (X1/3). The presence of ER in these clones significantly altered their growth characteristics, such that doubling times for the (i/ER) clones decreased in the presence of E2, but were higher in estrogen-depleted medium than those of the X1/3(i/neo) ER⁻ clone. Thus, the presence of ER in these cells allows E2-stimulated growth in monolayer culture. The results shown here demonstrate that E2 indeed has dual effects on ER⁺, HER-2/*neu*-overexpressing cancer cells; E2 will enhance transformation activity (measured by anchorage independence) but inhibit cell growth rate (measured by growth in monolayer).

4. Effects of Rb on Malignant Transformation Phenotypes of HER-2/*neu*-overexpressing Breast Cancer Cells

Using western blotting analysis, we have screened a panel of breast cancer cell lines for the non-expression of the Rb protein. We have identified MDA-MB-468 (breast cancer cell line) and SK-OV-3 (ovarian cancer cell line) that express aberrant form of the Rb protein. SK-OV-3 is known to overexpress HER-2/*neu* (35) but MDA-MB-468 express HER-2/*neu* at a basal level. We will continue to search for the Rb-defective and HER-2/*neu*-overexpressing breast cancer cells. At the same time, we will use SK-OV-3 ovarian cancer cell line as a model system to study the effect of Rb on malignant transformation phenotypes of HER-2/*neu*-overexpressing cancer cells since SK-OV-3 is Rb-defective and overexpresses HER-2/*neu*. To this end, the Rb-expressing vector has been co-transfected with pSV2-neo plasmid (10:1 molar ration between Rb and neo plasmid) into SK-OV-3 cells. The transfectants are currently under selection of G418 antibiotics. (The pSV2-neo will convert the recipient cells to become G418-resistant.)

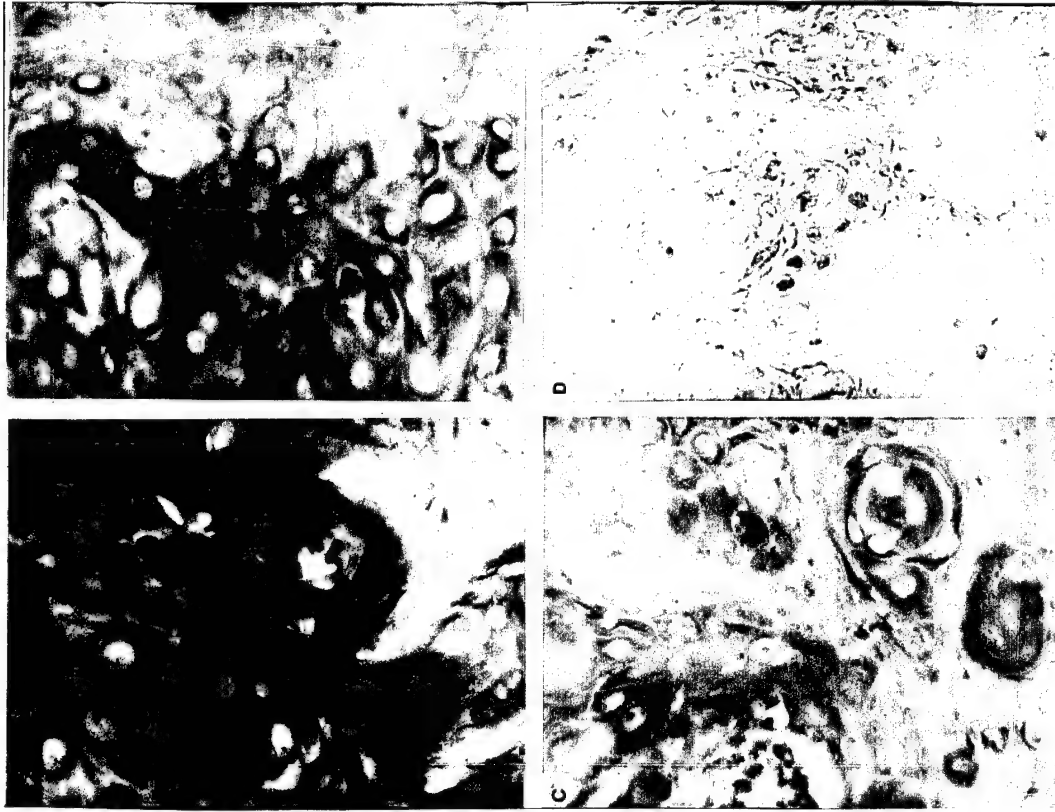


Figure 2. Immunohistochemical staining of HER-3.
The c-*erbB3*/HER-3 Ab-2 antibody (Oncogene Sciences, Inc.) was used to stain the oncoprotein;
A) high, B) intermediate, C) low, D) negative expression.
Magnification x400.

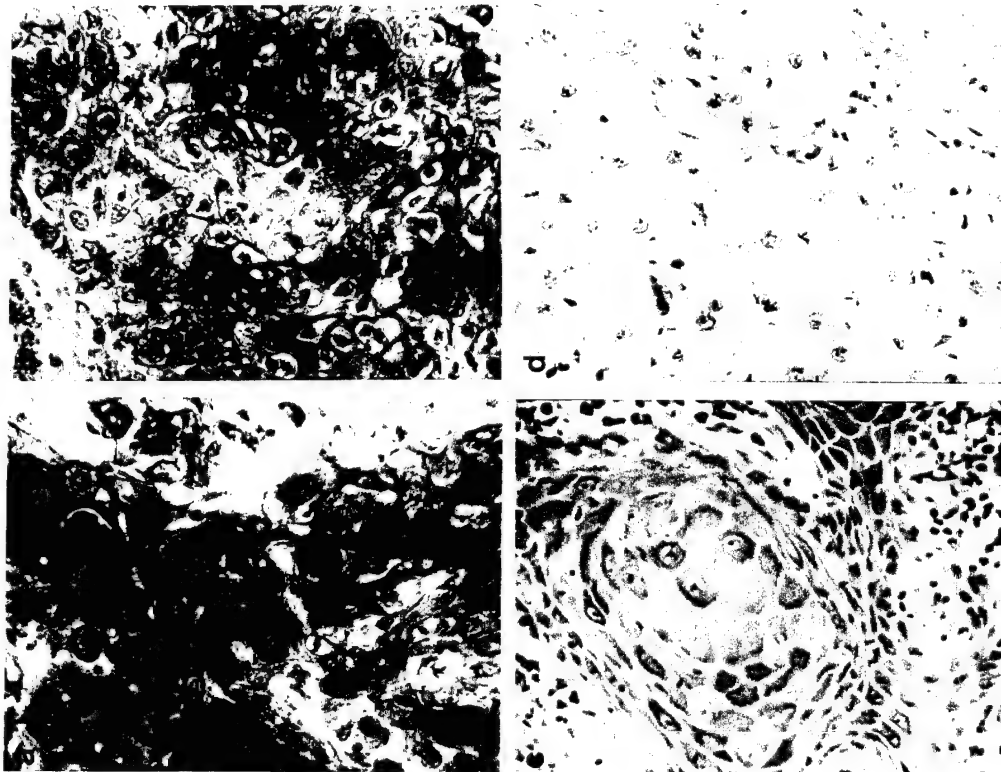


Figure 1. Immunohistochemical staining of HER-2/neu. The c-*neu* MAb 3 antibody (Oncogene Sciences, Inc.) was used to stain HER-2/*neu* encoded p185 protein from archival paraffin-embedded sections:
a) high, b) intermediate, c) low, d) negative expression.
Magnification x 400.

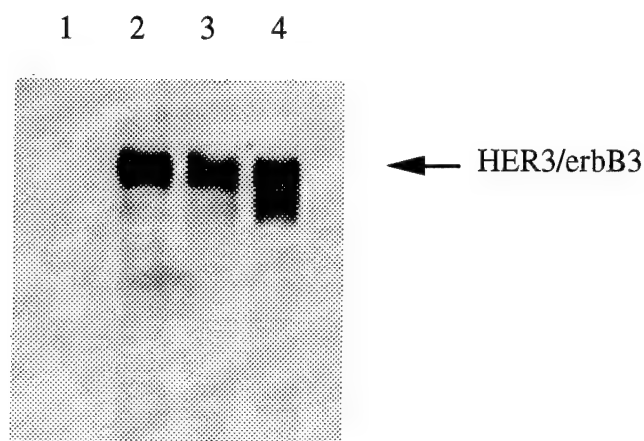


Figure 3. Western analysis of HER3/erbB3 in breast cancer cells.

50 μ g of lysates from different cell lines were separated on SDS/PAGE, followed by western blot with anti-HER3/erbB3 antibody. The position of HER3/erbB3 protein is indicated by an arrow. Lane 1, BT-20; lane 2, MDA-MB-231; lane 3, MDA-MB-435; lane 4, MDA-MB-453.

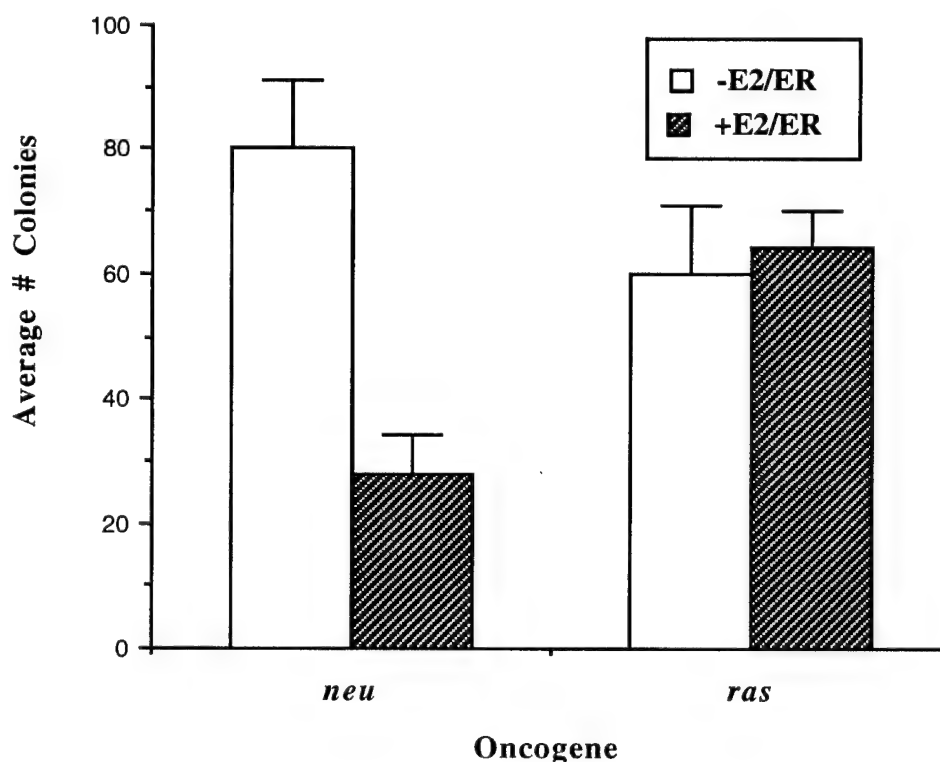


Figure 4. Focus formation by *HER-2/neu* and *ras*-transfected NIH/3T3 cells.

NIH/3T3 fibroblasts were transfected with either *HER-2/neu* or *ras* with or without ER. Plates from a single transfection were then split 1:4 and plated in the presence or absence of 10 nM E2. Foci of transformed NIH/3T3 cells are counted after 3-4 weeks of culture.

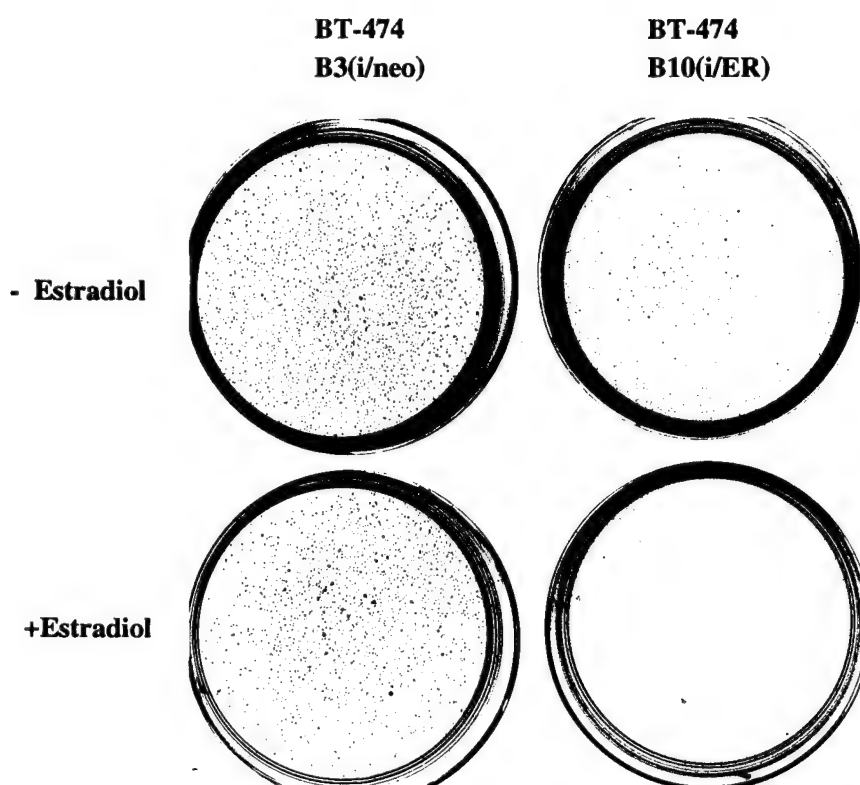


Figure 5. Soft agar colony formation by BT-474 subclones.

Subconfluent plates of each subclone were harvested and plated in agarose +/- 10 nM E2. Colonies were stained and counted manually after 3-4 weeks of culture. Data are summarized in Figure 6.

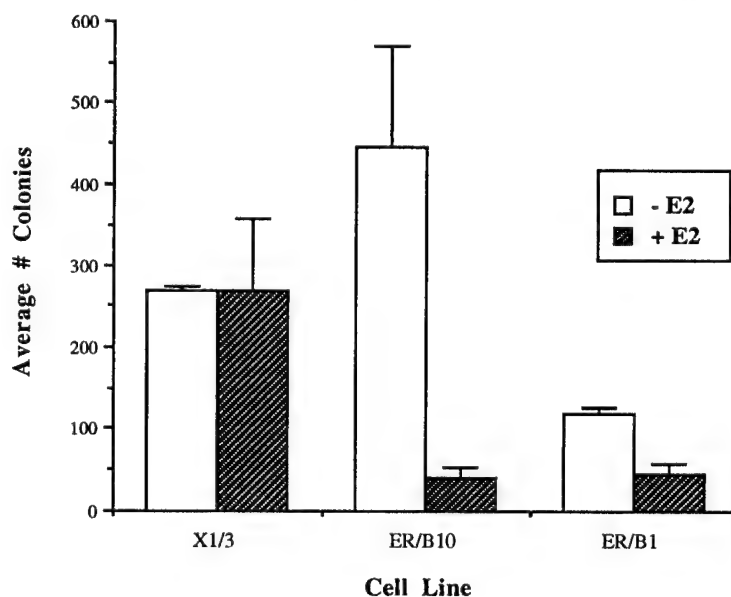


Figure 6. Summary graph of soft agar colony formation by BT-474 subclones.

Table I Focus formation by *HER-2/neu* and *ras* transfected NIH/3T3 cells

Oncogene vector	Avg # Foci (n=2)**	
	+ E2/ER	- E2/ER
c-nu 104 (<i>HER-2/neu</i>)	33	82
"	24	79
pT24-C3 (<i>ras</i>)	57	55
"	70	65

****Note that each row represents a single transfection, therefore data can only be compared across single rows. Direct comparisons between rows are invalid since transfection efficiency may alter the number of cells receiving the transforming vector. These data were normalized to form Figure 1 by measuring repression by estradiol in each transfection and comparing these values.**

Table II Doubling times of BT-474 subclones in monolayer culture

BT474 subclone	Doubling Time (days)		p
	+ Estradiol	- Estradiol	
X1/3(i/neo)	3.81	3.91	ns*
B10(i/ER)	3.24	4.70	<0.001
B1(i/ER)	2.89	4.43	<0.02

***ns: not significant**

CONCLUSION:

Task 1: Screening of 125 tumor sections for HER-2/*neu* and HER-3 has been completed. Due to the complexity caused by the newly identified HER-2 and HER-4 (82, 83), we will emphasize more on the staining of HER-2/*neu*, HER-3, HER-4 and Heregulin than Rb.

Task 2: Construction of Heregulin-expression vectors and anti-sense Heregulin plasmids have recently been completed. We have also identified several breast cancer cell lines that overexpresses HER-2/*neu* and also expresses HER-3. We will transfect Heregulin-expressing vector into these cells. Anti-sense construct will be transfected into MDA-MB-231 cells that are known to express Heregulin previously.

Task 3: Characterization of E2 on ER⁺, HER-2/*neu*-overexpressing breast cancer cells has been completed. The results, as described in the Body section, allow us to conclude that transformation of the breast cancer cells that express ER and overexpress HER-2/*neu* can be suppressed by the presence of estrogen. This is due to HER-2/*neu* expression can be repressed by estrogen-ER interaction. This conclusion was predicted in the original proposal and support the original hypothesis that cancer patients with HER-2/*neu* overexpression and ER⁺ breast tumor may not response to Tamoxifen. The effect of Tamoxifen on the E2/ER-mediated phenotypes will be examined as described in the original proposal.

Task 4: We have identified both MDA-MB-468 and SK-OV-3 cancer cell lines in which Rb gene is defective by screening Rb expression. Transfection of Rb expression vector into SK-OV-3 to establish Rb transfectants is currently under the selection procedure.

In addition to the those described in the BODY Section, several studies relating to HER-2/*neu* oncogene in breast cancer have been completed. The funding support from the current project has been appropriately acknowledged in the resulting publications. These include:

1. Lisha Zhang, Ching-jer Chang, Sarah S. Bacus, and **Mien-Chie Hung**. Suppressed transformation and induced differentiation of HER-2/*neu*-overexpressing breast cancer cells by emodin. *Cancer Res.* 55: 3890-3896, 1995.
2. Nobutaka Kiyokawa, Duen-Hwa Yan, Mary Elizabeth Brown, and **Mien-Chie Hung**. Cell Cycle-dependent regulation of p185^{neu}: a relationship between disruption of this regulation and transformation. *Proc. Natl. Acad. Sci. USA* 92: 1092-1096, 1995.
3. Susan J. Miller, and **Mien-Chie Hung**. Regulation of HER-2/*neu* gene expression. *Oncology Reports* 2: 497-503, 1995.
4. Youming Xie, Nobutaka Kiyokawa, Angabin Matin, and **Mien-Chie Hung** Signal transduction of HER-2/*neu* receptor tyrosine kinase *The Cancer Bulletin* 47: 109-118, 1995

Reprints or copies of these papers have been previously submitted.

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APPENDIX

Suppressed Transformation and Induced Differentiation of *HER-2/neu*-overexpressing Breast Cancer Cells by Emodin¹

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ABSTRACT

The amplification and overexpression of the *HER-2/neu* proto-oncogene, which encodes the tyrosine kinase receptor p185^{neu}, have been observed frequently in tumors from human breast cancer patients and are correlated with poor prognosis. To explore the potential of chemotherapy directed at the tyrosine kinase of p185^{neu}, we have found that emodin (3-methyl-1,6,8-trihydroxyanthraquinone), a tyrosine kinase inhibitor, suppresses autophosphorylation and transphosphorylation activities of *HER-2/neu* tyrosine kinase, resulting in tyrosine hypophosphorylation of p185^{neu} in *HER-2/neu*-overexpressing breast cancer cells. Emodin, at a 40- μ M concentration, which repressed tyrosine kinase of p185^{neu}, efficiently inhibited both anchorage-dependent and anchorage-independent growth of *HER-2/neu*-overexpressing breast cancer cells. However, the inhibition was much less effective for those cells expressing basal levels of p185^{neu} under the same conditions. Emodin also induced differentiation of *HER-2/neu*-overexpressing breast cancer cells by exhibiting a morphological maturation property of large lacy nuclei surrounded by sizable flat cytoplasm and by showing a measurable production of large lipid droplets, which is a marker of mature breast cells. Therefore, our results indicate that emodin inhibits *HER-2/neu* tyrosine kinase activity and preferentially suppresses growth and induces differentiation of *HER-2/neu*-overexpressing cancer cells. These results may have chemotherapeutic implications for using emodin to target *HER-2/neu*-overexpressing cancer cells.

INTRODUCTION

The proto-oncogene *HER-2/neu* (also known as *c-erbB-2*) encodes a 185-kilodalton (p185^{neu}) transmembrane tyrosine kinase growth factor receptor, which is related to but distinct from the EGFR³ (1-5). Amplification and overexpression of the *HER-2/neu* proto-oncogene occur in as many as 30% of breast cancers and have been found to be correlated with both poor prognosis (6-9) and decreased survival in breast cancer patients (6, 7). Furthermore, the results from cellular and animal experiments show that enhancement of *HER-2/neu* tyrosine kinase activity increases malignant phenotypes (10-17). These results suggested that the tyrosine kinase activity enhanced by *HER-2/neu* overexpression may play a critical role in the development of human tumors. Therefore, an inhibitor of the *HER-2/neu* tyrosine kinase activity may be able to repress cell transformation through repression of tyrosine kinase activity. To explore this possibility, we examined human breast cancer cells that overexpress the *HER-2/neu* oncogene and found that emodin (3-methyl-1,6,8-trihydroxyanthraquinone), an inhibitor for the protein tyrosine kinase p56^{lck} (18) isolated from *Polygonum cuspidatum*, inhibited activity of p185^{neu} tyrosine kinase,

preferentially blocked the growth of the *HER-2/neu*-overexpressing human breast cancer cells, and induced differentiation of these cells.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines MDA-MB453, BT-483, MDA-MB231, and MCF-7 and immortalized breast cell line HBL-100 were obtained from the American Type Culture Collection (Rockville, MD). AU-565 cells were obtained from the Naval Bioscience Laboratory (Oakland, CA). MDA-MB453, BT483, and AU-565 cells overexpress *HER-2/neu*, whereas MDA-MB231, MCF-7, and HBL-100 cells express basal levels of *HER-2/neu*. All cells were grown in DMEM/Ham's F12 (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and gentamicin (50 μ g/ml). Cells were grown in a humidified incubator at 37°C under 5% CO₂ in air.

Immunoprecipitation and Western Blot Analysis. As described previously (19, 20), cells were washed three times with PBS and then lysed in lysis buffer [20 mM Na₂PO₄ (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 100 mM NaF, and 2 mM Na₃VO₄]. Protein content was determined against a standardized control using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A total of 500 μ g of protein were immunoprecipitated with an anti-p185^{neu} mAb [c-*neu* (Ab-3); Oncogene Science, Inc., Uniondale, NY] on protein A-Sepharose. The immune complexes were washed, separated by 6% SDS-PAGE, and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Nonspecific binding on the nitrocellulose filter paper was minimized with a blocking buffer containing nonfat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS. The treated filter paper was incubated with primary antibodies [anti-p185^{neu} antibody c-*neu* (Ab-3) for detection of p185^{neu} or anti-PY antibody (Upstate Biotechnology, Inc., Lake Placid, NY) for detection of PY], followed by incubation with horseradish peroxidase-conjugated goat antimouse antibody (1:1000 dilution; Boehringer Mannheim Corp., Indianapolis, IN), and bands were visualized with the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Immunocomplex Kinase Assay. The immunocomplex kinase assay was modified from those described previously (21). In brief, cells were treated with or without 40 μ M emodin for 24 h and then washed three times with PBS. Cells were then collected and lysed in lysis buffer. Cell lysates (500 μ g) were incubated with anti-p185^{neu} mAb c-*neu* (Ab-3) for 1 h at 4°C, precipitated with 50 μ l of protein A-conjugated agarose (Boehringer Mannheim) for 30 min at 4°C, and washed three times with 50 mM Tris-HCl buffer containing 0.5 M LiCl (pH 7.5) and once in assay buffer [50 mM Tris-HCl (pH 7.5) and 10 mM MnCl₂]. To 40 μ l of beads (protein A-conjugated agarose), 10 μ l of [γ -³²P]ATP (Amersham) and 10 μ l of enolase (Sigma Chemical Co., St. Louis, MO) were added and incubated for 20 min at room temperature. The reactants were separated by 7.5% SDS-PAGE. The gel was dried and visualized by autoradiography.

Proliferation Assay. Cells were detached by trypsinization, seeded at 2×10^4 cells/ml in a 96-well microtiter plate overnight, treated with various concentrations of test samples, and incubated for an additional 72 h. The effect of emodin on cell growth was examined by MTT assay (22, 23). In brief, 20 μ l of MTT solution (5 mg/ml; Sigma) were added to each well and incubated for 4 h at 37°C. The supernatant was aspirated, and the MTT formazan formed by metabolically viable cells was dissolved in 150 μ l of DMSO then monitored by a microplate reader (Dynatech MR 5000 fluorescence; Dynatech Corp., Burlington, MA) at a wavelength of 590 nm.

Colony Formation in Soft Agarose. As described previously (14), cells (1×10^3 cells/well) were seeded in 24-well plates in culture medium containing 0.35% agarose (FMC Corp., Rockland, ME) over a 0.7% agarose layer and incubated for 3 weeks at 37°C. Colonies were then stained with *p*-iodonitrotet-

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³ The abbreviations used are: EGFR, epidermal growth factor receptor; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PY, phosphotyrosine.

razoium violet (1 mg/ml), and colonies larger than 100 μ m were counted. Each determination was performed four times.

Lipid Visualization. As described previously (24), a modified Oil Red O in propylene glycol method was used to visualize neutral lipids (25, 26).

RESULTS

Effect of Emodin on Tyrosine Phosphorylation in Breast Cancer Cells That Overexpress *HER-2/neu*. To test whether emodin, a tyrosine kinase inhibitor for the protein tyrosine kinase p56^{lck} (18), also inhibits *HER-2/neu* tyrosine kinase, MDA-MB453 human breast cancer cells that overexpress p185^{neu} were used to test the effect of emodin on tyrosine phosphorylation of p185^{neu}. Cells were treated with varying concentrations of emodin at 37°C for 24 h then analyzed for the protein level of p185^{neu} and its tyrosine phosphorylation. The p185^{neu} was first immunoprecipitated by anti-p185^{neu} antibody, and the immunoprecipitates were then blotted with anti-PY antibody for detection of PY or anti-p185^{neu} antibody for detection of p185^{neu}. Emodin, at a 40- μ M concentration, induced a significant reduction in the level of tyrosine phosphorylation (Fig. 1A) but had no obvious effect on p185^{neu} protein level. The reduced tyrosine phosphorylation of p185^{neu} could be detected readily after 12 h (Fig. 1B). To confirm further that the reduced tyrosine phosphorylation by emodin is a general phenomenon for p185^{neu}, other *HER-2/neu*-overexpressing breast cancer cell lines were also examined, and similar results were obtained (Fig. 2). These cell lines include BT-483, AU-565 (Fig. 2), SKBr-3, and MDA-MB361 (data not shown). When MCF-7 cells that express basal levels of p185^{neu} protein were examined similarly, the PY level of p185^{neu} was almost undetectable under our experimental condition. Therefore, it is insignificant to compare the effect of emodin on PY level on p185^{neu} in MCF-7 cells (data not shown).

Repression of Autophosphorylation and Transphosphorylation by Emodin *in Vitro*. The results shown above indicated that PY level of p185^{neu} could be repressed by emodin. To examine whether the reduced tyrosine phosphorylation could affect the tyrosine kinase activity of p185^{neu}, we carried out the immunocomplex kinase assay. The autophosphorylation ability of p185^{neu} from MDA-MB453 cells treated with emodin for 24 h was inhibited, and the transphosphorylation ability of p185^{neu} for enolase, an exogenous substrate for

tyrosine kinase, also was significantly decreased compared with that of untreated cells (Fig. 3A). These data (Figs. 1 and 3) indicated that emodin-treated cells result in reduced PY levels in p185^{neu}, which, in turn, exhibits lower tyrosine kinase activity. To address further whether emodin can directly inhibit the intrinsic tyrosine kinase activity of p185^{neu}, p185^{neu} was immunoprecipitated from the untreated MDA-MB453 cells; the precipitates were then treated with varying concentrations of emodin, and the kinase activity was measured. The tyrosine kinase activity for both autophosphorylation and transphosphorylation of p185^{neu} is inhibited by emodin in a dose-dependent manner (Fig. 3B). These results show conclusively that emodin represses the intrinsic tyrosine kinase activity of p185^{neu}. Therefore, the reduced PY level in p185^{neu} treated with emodin is most likely caused by inhibition of p185^{neu} tyrosine kinase activity.

Effect of Emodin on the Proliferation of Human Breast Cancer Cells. Because emodin effectively inhibits the tyrosine kinase activity of p185^{neu}, which is critical for cell growth, it is of interest to investigate whether emodin may inhibit cell proliferation preferentially for the breast cancer cells that overexpress p185^{neu}. To address this issue, we chose six cell lines for additional study. MDA-MB453, BT-483, and AU-565 are *HER-2/neu*-overexpressing breast cancer cell lines, as mentioned above, and MCF-7 and MDA-MB231 are two human breast cancer cell lines that express basal levels of p185^{neu} (27). The HBL-100 cell line is derived from a normal human breast tissue transformed by SV40 large T antigen (43) and expresses a basal level of p185^{neu}. As shown in Fig. 4, the growth of these cells was inhibited by emodin in a dose-dependent manner but to varying extents. At a 40- μ M concentration, which effectively inhibited tyrosine kinase activity of p185^{neu} (Figs. 1, 2, and 3), emodin blocked 68, 72, and 84% of growth in MDA-MB453, BT-483, and AU-565 cells, respectively. However, under the same conditions, it inhibited only 37 and 23% of growth in MCF-7 and MDA-MB 231 cells, respectively. Emodin had little effect on HBL-100 breast cells even up to an 80- μ M concentration. The differential growth effect was not obvious when the cells were treated with 10 μ M emodin, which did not affect tyrosine kinase activity of p185^{neu}. These results indicate that emodin preferentially suppresses growth of the *HER-2/neu*-overexpressing

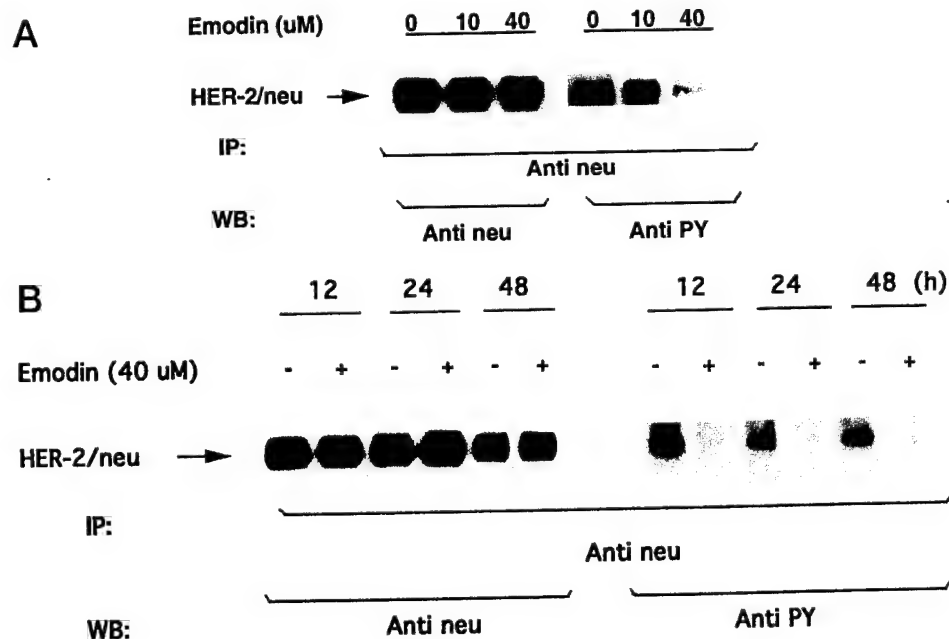


Fig. 1. Effect of dose dependence and time course of emodin treatment on tyrosine phosphorylation and expression of *HER-2/neu* in MDA-MB453 cells. A, cells in the serum-free medium were incubated without (0) or with emodin (10 or 40 μ M) at 37°C for 24 h. B, cells were incubated without (–) treatment or with (+) emodin (40 μ M) at 37°C for varying times. Cell extracts were immunoprecipitated by anti-p185^{neu} antibody (Anti neu) and then Western blotted with either anti-PY antibody (Anti PY) or anti-p185^{neu} antibody, as described in “Materials and Methods.”

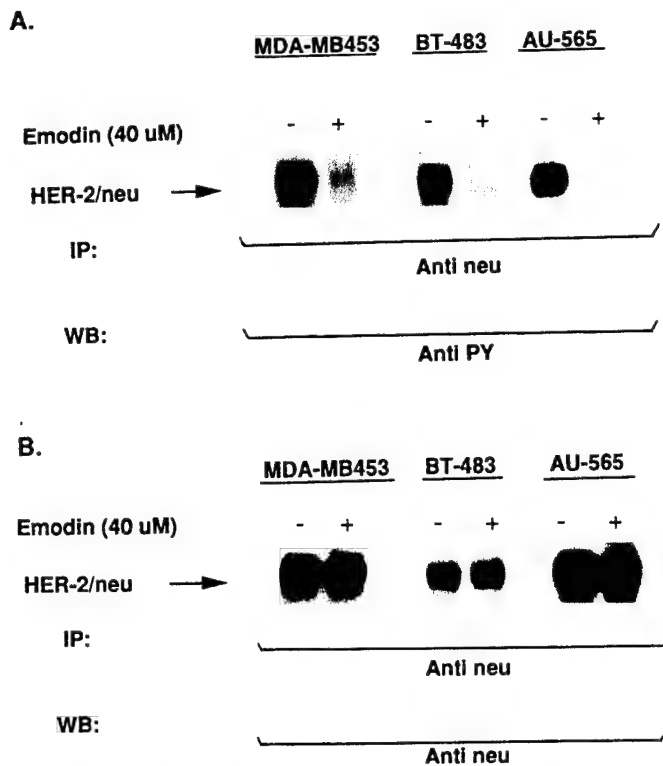


Fig. 2. Effect of emodin on tyrosine phosphorylation and expression of *HER-2/neu* in human *HER-2/neu*-overexpressing breast cancer cells. Cells in the serum-free medium were incubated without (-) or with (+) 40 μ M emodin at 37°C for 24 h, and then cell lysates were immunoprecipitated by anti-p185^{neu} antibody and blotted with anti-PY antibody (A) or anti-p185^{neu} antibody (B), as described in "Materials and Methods."

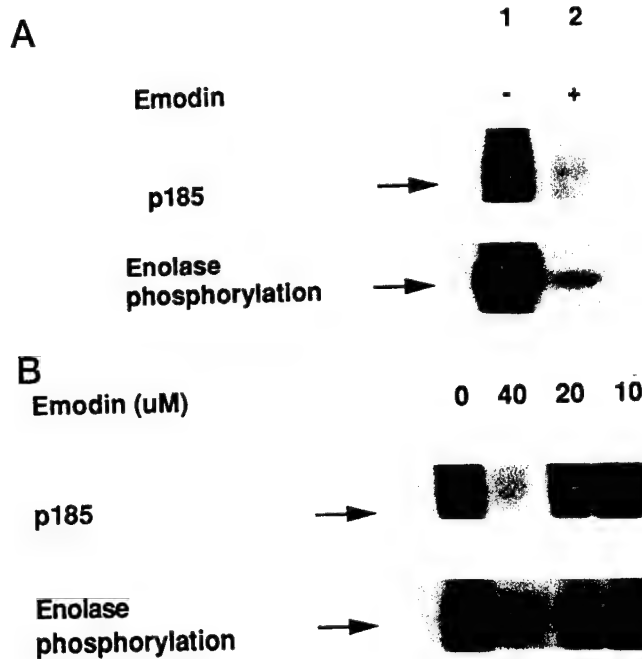


Fig. 3. Effect of emodin on autophosphorylation and transphosphorylation of P185^{neu} in MDA-MB453 breast cancer cells. A, cells were incubated without (Lane 1) or with (Lane 2) emodin (40 μ M) at 37°C for 24 h, then cell lysates (500 μ g) were immunoprecipitated, and kinase activities were measured by incubation with [γ -³²P]ATP and enolase. B, cell lysates from untreated cells were immunoprecipitated then incubated with [γ -³²P]ATP, enolase, and varying concentrations of emodin for 20 min at room temperature. Reactants were resolved on 7.5% SDS-PAGE. The phosphorylation products were dried and visualized by autoradiography, as described in "Materials and Methods."

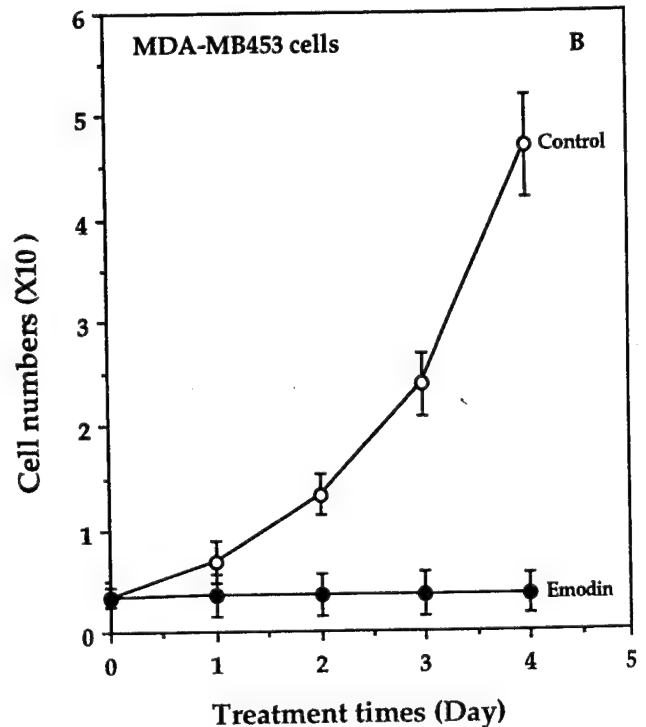
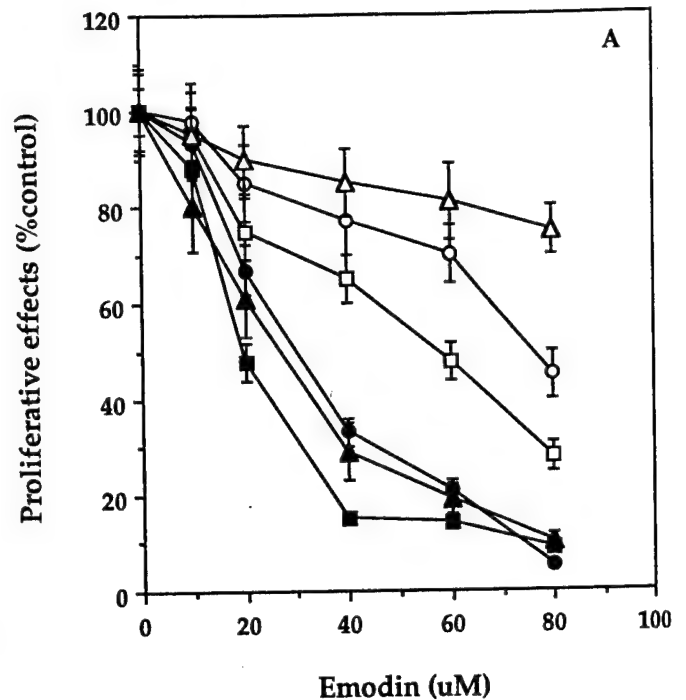


Fig. 4. Effect of emodin on the proliferation of human breast cancer cells expressing different levels of *HER-2/neu*. A, MDA-MB453 (●), AU-565 (▲), and BT-483 cells (■), which overexpress *HER-2/neu*, and MCF-7 (□), MDA-MB231 (○) and HBL-100 cells (△), which express normal levels of *HER-2/neu*, were incubated without or with different concentrations of emodin at 37°C for 72 h. The effect on cell growth was examined by MTT assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment of emodin as 100%. B, MDA-MB453 cells were incubated without (control) or with emodin (40 μ M) at 37°C for different times, and cells were washed and counted by trypan blue exclusion with hemacytometer. All determinations were made in triplicate. Bars, SD.

breast cancer cells and suggest that the differential suppression effect is likely to occur through repression of p185^{neu} tyrosine kinase.

Because emodin at a 40- μ M concentration inhibited p185^{neu} tyrosine kinase activity (Figs. 1, 2, and 3) and significantly suppressed

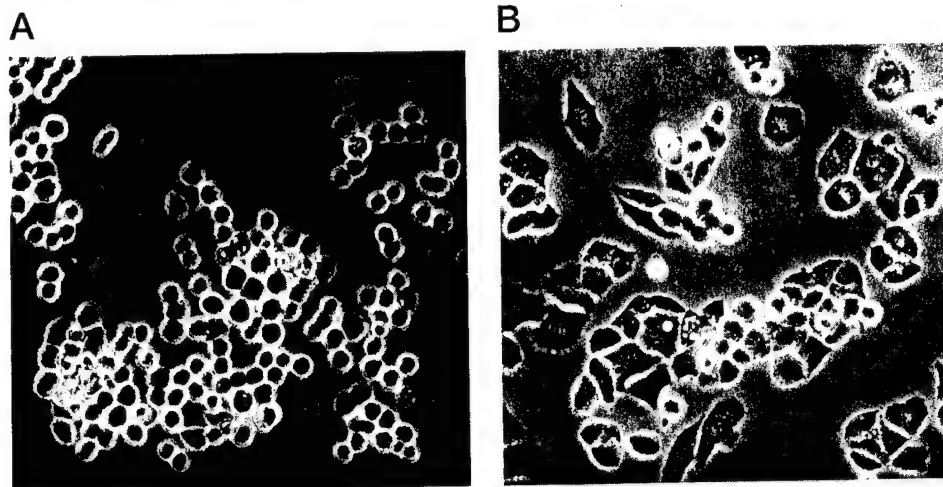


Fig. 5. Effect of emodin on morphology of MDA-MB453 human breast cancer cells. Cells were incubated without (A) or with 40 μ M emodin (B) for 24 h. The change in cell morphology was examined and photographed under an inverted microscope.

growth of breast cancer cells that overexpress p185^{neu} (Fig. 4A), we examined further the effect of time course on proliferation of MDA-MB453 cells by emodin at a 40- μ M concentration. Emodin inhibited cancer cell growth completely, and, when the viability of cells was measured by trypan blue assay, more than 90% of cells were found to be alive (Fig. 4B). The results suggest that the mechanism that causes inhibition of cell growth is primarily a result of repression of proliferation and not induction of cell death under this condition.

Emodin Induces Differentiation of Breast Cancer Cells. Because emodin inhibited breast cancer cell growth, and no significant cell deaths were observed (Fig. 4B), it was of interest to investigate whether emodin would induce differentiation of breast cancer cells. When MDA-MB453 cells were treated with emodin (40 μ M) for 24 h, cells displayed a flat morphology with larger nuclei and increased cytoplasm, which were shown to be characteristic for differentiation (28; Fig. 5B), compared with untreated cells, which are moderately adherent and have a rounded morphology (Fig. 5A). A similar morphological change was also observed in AU-565 cells (data not shown). In contrast, no significant morphological change could be observed in the MCF-7 cells treated by emodin compared with untreated cells (data not shown).

Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined further whether the morphological change induced by emodin was accompanied by the production of lipid droplets. Large droplets containing neutral lipid were readily detectable in emodin-treated cells (Fig. 6, B and D); in contrast, no obvious large lipid droplets could be observed in the untreated cells (Fig. 6, A and C). When the lipid-producing cells were counted, more than 90% of the emodin-treated cells produced large lipid droplets, but only 2 to 5% of the untreated cells contained lipid drops of a much smaller size. In contrast, no obvious large lipid droplets could be observed in the MCF-7 cells treated with 40 μ M emodin (data not shown). These results indicate that emodin preferentially induces differentiation of *HER-2/neu*-overexpressing breast cancer cells, suggesting that the enhanced tyrosine kinase activity of p185^{neu} may prevent breast cancer cells from differentiation.

Effect of Emodin on Transformation of Breast Cancer Cells. One hallmark of the transformed state is the ability of cells to exhibit anchorage-independent growth. To determine whether emodin may affect this property in breast cancer cells, cells were seeded into soft agarose and monitored for colony formation (Fig. 7). The colony-forming activity of *HER-2/neu*-overexpressing breast cancer cells MDA-MB453, BT483, and AU565 in soft agarose containing 40 μ M emodin was suppressed dramatically. However, under the same

condition, the cells that express basal levels of p185^{neu}, namely MCF-7, MDA-MB231, and HBL-100, still formed a significant number of colonies. The decreased ability to grow in soft agarose of the *HER-2/neu*-overexpressing breast cancer cells could not simply reflect the slower proliferation rate shown in Fig. 4, because 3 additional weeks of incubation did not increase the number of colonies. Furthermore, no significant change in colony-forming activity could be observed when the cells grew in soft agarose containing 10 μ M emodin (data not shown), which did not inhibit tyrosine kinase activity of p185^{neu} (Figs. 1 and 3B). Taken together, the results indicate that emodin preferentially suppresses the transformation ability of *HER-2/neu*-overexpressing cancer cells and suggest that the transformation suppression by emodin may be mediated through its ability to inhibit tyrosine kinase activity of p185^{neu}.

DISCUSSION

Emodin has been reported to be a tyrosine kinase inhibitor that restricts the activity of p56^{lck} kinase by preventing the binding of ATP *in vitro* (18) and that has the ability to inhibit the growth of cancer cells, including lymphocytic leukemia (29), HL-60 human leukemia cells (30), and *ras*-transformed human bronchial epithelial cells (31), by an unknown mechanism. In this study, we demonstrated that emodin suppressed tyrosine kinase activity of *HER-2/neu*, inhibited cell growth and the transformation phenotype *in vitro* for human breast cancer cells that overexpress the *HER-2/neu* oncogene, and induced differentiation of these cells.

Tyrosine kinase receptors play a vital role in the cascade of signals leading to cell growth and differentiation (32). It has been shown previously that several agents, including a specific mAb against p185^{neu} (26), mycophenolic acid, and phorbol ester (25), can induce differentiation of breast cancer cells through down-regulation or translocation of p185^{neu} to cytoplasmic and nuclear sites. In this study, we report that emodin, which suppresses tyrosine kinase activity of p185^{neu} without changing the p185^{neu} protein level, can inhibit both anchorage-dependent and anchorage-independent cell growth and induce the mature phenotype. These results suggest further that the tyrosine kinase activity of p185^{neu} is critical for cell growth and *HER-2/neu*-induced transformation, and that repression of the kinase activity may also induce differentiation.

The p185^{neu} transmembrane glycoprotein with intrinsic tyrosine kinase activity is homologous to EGFR (2, 4, 5). To examine whether the inhibitor of EGFR tyrosine kinase affects the tyrosine kinase activity of p185^{neu}, we also tested the effect of genistein, a specific inhibitor of the EGFR tyrosine kinase (33), on tyrosine phosphoryl-

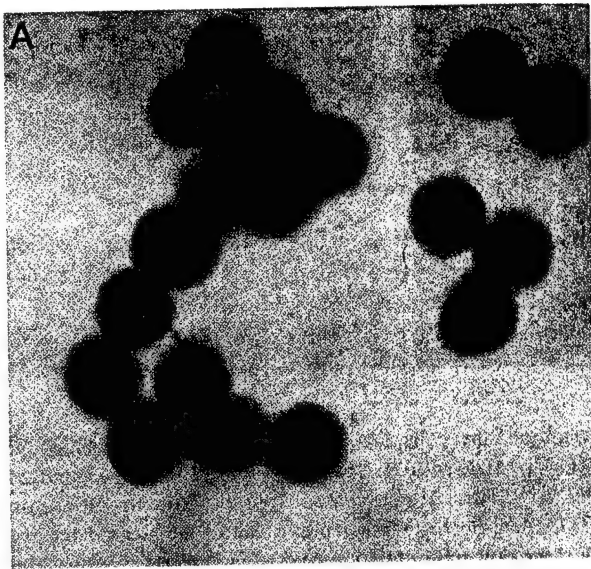
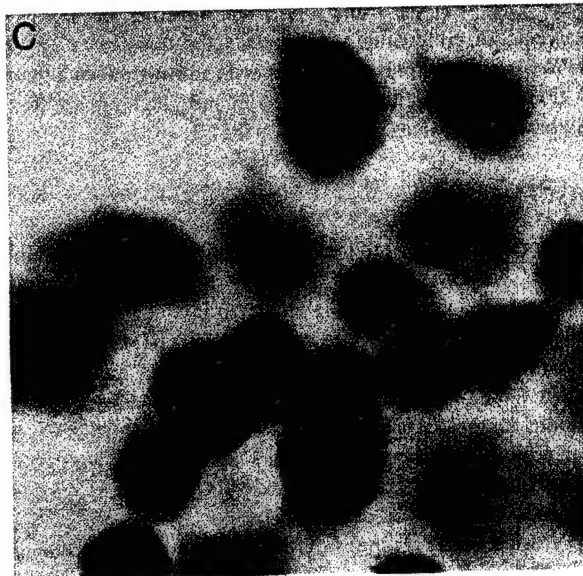
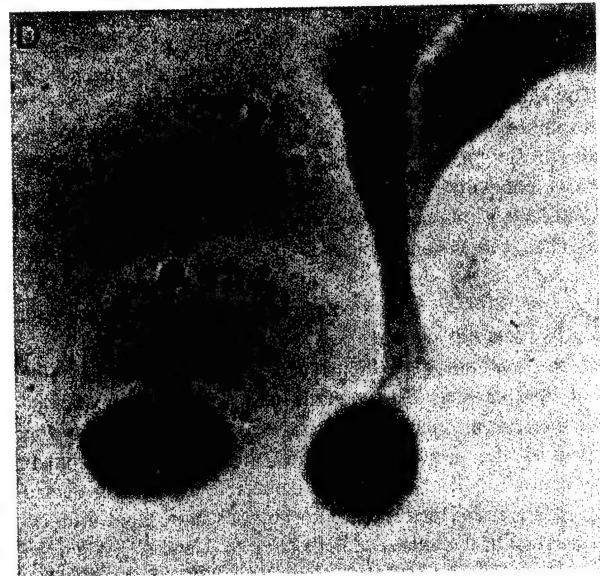
MDA-MB453 cells**Untreated****Emodin****AU-565 cells****Untreated****Emodin**

Fig. 6. Emodin-induced appearance of neutral lipid droplets in MDA-MB453 and AU-565 cells. Cells were treated without (A and C) or with (B and D) emodin (40 μ M) for 5 days then processed for visualization of neutral lipids as described in "Materials and Methods."

ation of p185^{neu} in MDA-MB453 cells that express high levels of p185^{neu} and basal levels of EGFR (27, 34). Genistein, at a concentration (6 μ g/ml) known to inhibit tyrosine kinase activity of EGFR (33), had no obvious effect on tyrosine phosphorylation of p185^{neu} (data not shown). Although additional study is needed, these results suggest that different tyrosine kinase receptors may respond differently to different tyrosine kinase inhibitors. To prevent complications from the interaction between EGFR and p185^{neu}, the cell lines used in this study express basal levels of EGFR only (34).

Another interesting issue for the emodin-induced effect on *HER-2/neu*-overexpressing cells is whether the effect is reversible. MDA-MB453 cells were treated with 40 μ M emodin for 1 day in the absence of serum, emodin was removed, and the cells were incubated in the

presence of serum with or without emodin for 2 more days; we found that, in the absence of emodin for two more days, both the PY level of p185^{neu} and the growth rate of the cells were significantly higher than those in the presence of emodin (data not shown). The results suggest that the suppression effect of emodin on cell proliferation and PY level of p185^{neu} may not be irreversible under the conditions described above.

The autophosphorylation of tyrosine kinase receptors is believed to be the first step in signal transduction and a prerequisite for the phosphorylation of downstream targets by the receptors (35–37). The p185^{neu} protein has been shown previously to be associated with and to phosphorylate phospholipase C- γ , the GTPase-activating protein GAP, phosphatidylinositol 3'-kinase, and Shc (38–42). Activation of

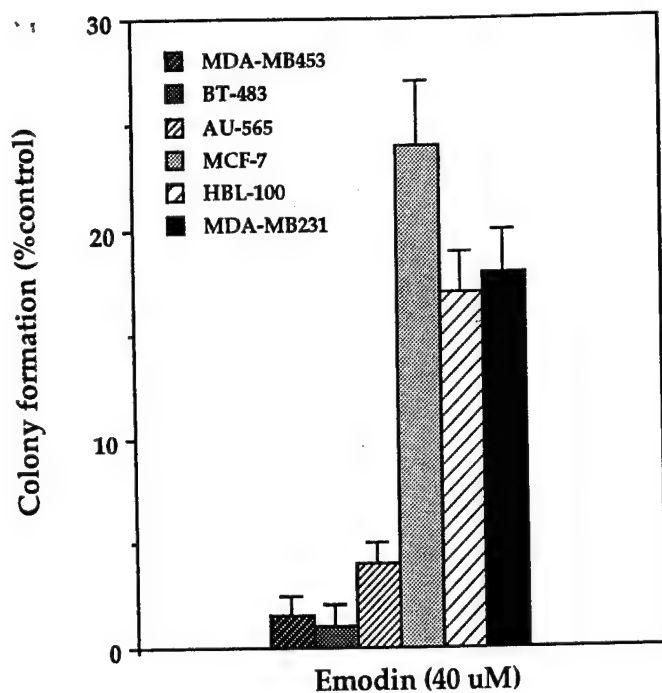


Fig. 7. The effect of emodin on human breast cancer cell colony growth in soft agarose. Cells (1×10^3 cells/well) were seeded into 24-well plates in culture medium containing 0.35% agarose over a 0.7% agarose layer with or without 40 μ M emodin and incubated for 3 weeks at 37°C. Colonies were stained with *p*-iodonitrotetrazolium violet and counted, and the percentage of colony formation was calculated by defining the number of colonies in the absence of emodin as 100%. All determinations were made four times. Bars, SD.

these downstream targets by phosphorylation requires tyrosine kinase activity of p185^{neu}. Because intrinsic tyrosine kinase activity of p185^{neu}, which triggers both autophosphorylation and transphosphorylation, is effectively repressed by emodin (Fig. 3), these downstream targets are not likely to be activated. It is known that *ras* plays a role in one of the downstream targets (38–42). Because emodin was shown to inhibit growth of *ras*-transformed cells (31), inactivation of the *ras* pathway might contribute to the growth suppression of *HER-2/neu*-overexpressing breast cancer cells. However, as shown in this study, emodin can affect multiple biological properties of *HER-2/neu*-overexpressing breast cancer cells, including suppression of anchorage-dependent and anchorage-independent growths and induction of differentiation. It will be of interest to investigate further how the different downstream targets of p185^{neu} may trigger cell growth and differentiation. In addition, because emodin preferentially inhibits growth of cancer cells with *HER-2/neu*-overexpression, it may have therapeutic implications for cancers with *HER-2/neu* overexpression.

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Cell cycle-dependent regulation of p185^{neu}: A relationship between disruption of this regulation and transformation

(*HER2/c-erbB-2*/growth factor receptor)

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ABSTRACT Structure and function of p185^{neu} receptor tyrosine kinase were found to be regulated in a cell cycle-dependent manner. In M phase, p185^{neu} is hyperphosphorylated at serine and/or threonine residues. The phosphotyrosine [Tyr(P)] content of p185^{neu} is at its highest level in G₀/G₁ phase, decreases through S and G₂ phases, and reaches its lowest level in M phase. Phospholipase C-γ (PLC-γ) and GTPase-activating protein (GAP), substrates of p185^{neu}, also have a similar profile of Tyr(P) content during the cell cycle. These results, along with *in vitro* immune complex kinase assays, suggest that the tyrosine kinase activity of p185^{neu} is least active in M phase. Interestingly, the mutation-activated *neu* oncogene (*neu*^{*})-encoded protein product, p185^{neu*}, escaped from cell cycle regulation. Taken together, we demonstrate in this report that the structure and function of p185^{neu} are regulated in a cell cycle-dependent manner, yet p185^{neu*} escapes from this regulation and remains active through the cell cycle. Disruption of this cell cycle regulation may define a mechanism for p185^{neu*}-mediated cellular transformation.

The 185-kDa protein product of the *neu* (also called *c-erbB-2*, *HER2*, *NGL*, or *TKR1*) protooncogene, p185^{neu}, is a member of the growth factor receptor tyrosine kinase family and is closely related to the epidermal growth factor receptor (1–7). Recent studies have suggested that the signals raised by p185^{neu} are transduced through second messenger-producing proteins, such as phospholipase C-γ (PLC-γ), GTPase-activating protein (GAP), phosphatidylinositol 3'-kinase, and Shc (8–10). Although several ligands for p185^{neu} have been described recently (11–15), the true function of p185^{neu} remains unclear. It is known that a single point mutation in the transmembrane region (Val-664 to Glu) of *neu* converts the *neu* protooncogene into a transforming *neu* oncogene (*neu*^{*}) (5–7, 16, 17). The mutation-activated p185^{neu*} is associated with higher tyrosine kinase activity and exhibits potent transforming ability compared with the normal p185^{neu}. It is not yet clear how a single point mutation can enhance tyrosine kinase and transforming activities.

Receptor tyrosine kinases are known to stimulate proliferation of cells. Mitogenic signals are believed to promote the transition of cells from G₀ phase to G₁ phase and/or from G₁ phase to S phase in the cell cycle (18). Therefore, most cell cycle-related studies involving receptor tyrosine kinases focus on the transition from G₀ to G₁ or from G₁ to S phase. However, the structures and activities of growth-related molecules, such as Src, Abl, retinoblastoma gene product, and p53, which are important in regulating cell proliferation, have been shown to be regulated throughout the cell cycle (19–25). In an attempt to understand the role of p185^{neu} receptor tyrosine kinase in the cell cycle, we have investigated the structure and function of p185^{neu} throughout the cell cycle. The activity and structure of the p185^{neu} protein was found to be tightly

regulated through the entire cell cycle; however, the p185^{neu*} protein escaped from cell cycle regulation. This finding provides a plausible mechanism for the increased tyrosine kinase activity and potent transforming ability of p185^{neu*}.

MATERIALS AND METHODS

Cell Culture and Synchronization Procedures. Cell lines expressing normal p185^{neu}, DHFR/G8 or mutation-activated p185^{neu*}, B104-1-1, and SWX-1 have been described (1, 4, 26). All cell lines were grown in Dulbecco's modified Eagle's medium/nutrient mixture F-12 medium supplemented with 10% fetal calf serum.

To arrest in G₀/G₁ phase, cells were starved in medium with 0.5% serum for 48 hr (22). To accumulate in pre-S phase and G₂ phase, cells were incubated with aphidicolin and Hoechst 33342, respectively, as described (27). To synchronize in M phase, cells with the highly rounded mitotic morphology after nocodazole treatment, representative of cells that are in mid-to late M phase (prometaphase to telos) (28), were collected by mechanical shakeoff (19). For DHFR/G8 cells, the adherent cells remaining after nocodazole treatment that had a monolayer appearance with clear nuclei, representative of cells in G₂ and early M (prophase) (28), were also collected and examined. To confirm the actual cell synchronization, DNA content analysis was performed as described (19).

Protein Analysis. Preparation of cell lysates, SDS/PAGE, immunoblot analysis, immunoprecipitation, and *in vitro* immune complex kinase assay were performed as described (6, 9, 19). The total protein concentration of each cell lysate was determined by using the Bio-Rad protein assay kit. Immunoblots were developed by the enhanced chemiluminescence technique (ECL; Amersham) (9).

RESULTS

Structural Modification of p185^{neu} in the Cell Cycle. To first determine whether p185^{neu} might be regulated in a cell cycle-dependent manner, we examined potential cell cycle-dependent modifications of the protein. When protein extracts from DHFR/G8 cells, a nontransformed NIH 3T3 transfectant expressing high levels of normal p185^{neu} (4) in different stages of the cell cycle, were examined by immunoblotting with anti-p185^{neu} antibody, p185^{neu} migrated through SDS/PAGE in a cell cycle-specific fashion; p185^{neu} showed the fastest migration in G₀/G₁ phase and the most retarded migration in M phase. From pre-S through G₂ phases, p185^{neu} showed intermediate migration to the G₀/G₁ and M phase migration levels (Fig. 1*A* and *B*). When p185^{neu} from NIH 3T3 cells was examined, its mobility throughout the cell cycle was similar to

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Abbreviations: PLC-γ, phospholipase C-γ; GAP, GTPase-activating protein.

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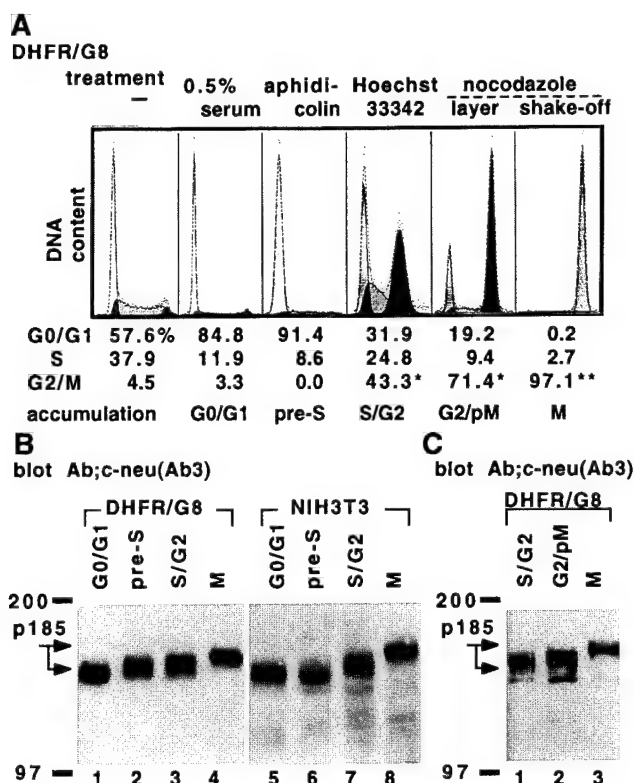


FIG. 1. Cell cycle-dependent migration shift of p185^{neu}. Both DHFR/G8 cells and NIH 3T3 cells were chemically synchronized in each cell cycle stage. (A) DNA content of DHFR/G8 cells was examined to confirm cell cycle synchronization. DNA histogram pattern, cell population in each cell cycle stage, and presumed synchronization are indicated for each treatment. It was also confirmed that DHFR/G8 cells and NIH 3T3 cells achieved similar cell cycle synchronizations (data not shown). *, Adherent cells; **, shakeoff cells; G2 to early M phase is indicated as G2/pM; mid- to late M phase is indicated as M. (B) Potential structural change of p185^{neu} during the cell cycle was examined by immunoblot analysis. After each treatment, cells were lysed and separated on a SDS/6% polyacrylamide gel and then subjected to immunoblot analysis with monoclonal anti-p185^{neu} antibody c-neu(Ab-3) (Oncogene Science). Lanes: 1–4, DHFR/G8 cells in each cell cycle stage (10 μ g per lane, 0.5-min exposure after reaction with ECL reagent); 5–8, NIH 3T3 cells (100 μ g per lane, 5-min exposure). Positions of p185^{neu} and molecular size markers (kDa) are indicated on the left. (C) Migration shift of p185^{neu} occurs in mid- to late M phase. Two populations from nocodazole-treated DHFR/G8 cells, shakeoff cells that arrested in mid- to late M phase (indicated as M; lane 3), and adherent cells that accumulated in G2 to early M phase (indicated as G2/pM; lane 2) were compared by immunoblotting as described in B. As a control, Hoechst 33342-treated DHFR/G8 cells, which accumulated in S to G2 phase, were also tested on the same gel (lane 1). Ab, antibody.

that of DHFR/G8 cells (Fig. 1B), indicating that the differential migration of p185^{neu} throughout the cell cycle is a general phenomenon for p185^{neu} and is not specifically caused by high-level expression of p185^{neu} in DHFR/G8 cells. To examine in more detail the migration shift of p185^{neu} in M phase, two different populations from nocodazole-treated DHFR/G8 cells, shakeoff cells (arrested in mid- to late M phase; prometaphase to telos) and adherent cells (accumulated in G2 phase to early M phase; prophase), were compared by immunoblotting. Cells in mid- to late M phase clearly displayed a significant migration shift, indicating that the migration shift occurred after prophase (Fig. 1C). In contrast, when B104-1-1 cells, a transforming NIH 3T3 transfectant containing neu* (1), were examined by the same procedure, p185^{neu} migrated in two forms (a high molecular weight form and a low molecular weight form) consistently throughout the

cell cycle with no significant difference in the mobility at different stages of the cell cycle (Fig. 2). To determine whether this phenomenon is specific for transforming p185^{neu}*, we examined protein from the SWX-1 cell line, a Swiss-Webster 3T3 cell line transformed by p185^{neu}* (26). The same result as for B104-1-1 cells was observed (Fig. 2B), suggesting that the cell cycle-independent pattern is a typical feature of transforming p185^{neu}*. All of the above data indicate that normal p185^{neu} undergoes cell cycle-dependent modifications, but mutated p185^{neu}* is independent of this regulation.

Functional Regulation of p185^{neu} in the Cell Cycle. We next wondered whether p185^{neu} function might also be regulated in a cell cycle-dependent manner. Since p185^{neu} is known to function as a tyrosine kinase (6, 7), we examined the intrinsic tyrosine kinase activity of p185^{neu} in different cell cycle stages by an *in vitro* immune complex kinase assay. In Fig. 3, we showed that the autophosphorylation ability of p185^{neu} from DHFR/G8 cells gradually decreased from G0/G1 phase to M phase. Also, the transphosphorylation ability of p185^{neu} for enolase, an exogenous substrate for tyrosine kinases, behaved similarly. When normal mouse serum was used as a control for the *in vitro* immune complex kinase assay, no significant transphosphorylation could be detected in the enolase (data not shown). These results suggest that the intrinsic tyrosine kinase activity of p185^{neu} is regulated in a cell cycle-specific manner. However, when we tested p185^{neu}* from B104-1-1 cells, we were not able to see a significant difference through the cell cycle for both auto- and transphosphorylation ability. This further suggests that the tyrosine kinase activity of p185^{neu}* escapes from cell cycle-dependent regulation. To examine whether the tyrosine kinase activity of p185^{neu} indeed

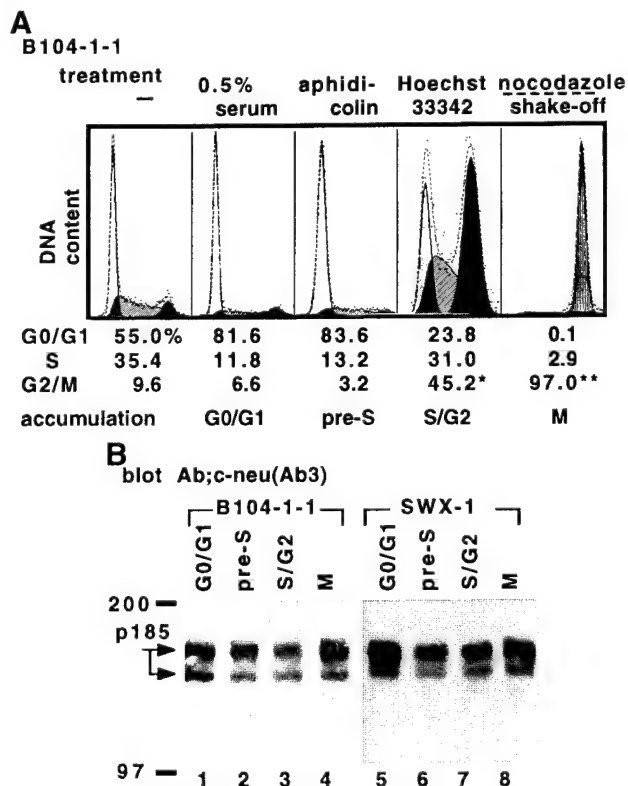


FIG. 2. No significant migration shift of p185^{neu}* during the cell cycle. (A) B104-1-1 cells and SWX-1 cells were chemically synchronized in each cell cycle stage as described in Fig. 1. The result of DNA content analysis of B104-1-1 cells was presented. (B) Potential structural change of p185^{neu}* during the cell cycle was examined by immunoblot analysis similar to the procedure described in Fig. 1B. Lanes: 1–4, B104-1-1 cells in each cell cycle stage (25 μ g per lane); 5–8, SWX-1 cells (25 μ g per lane). Numbers on left are kDa.

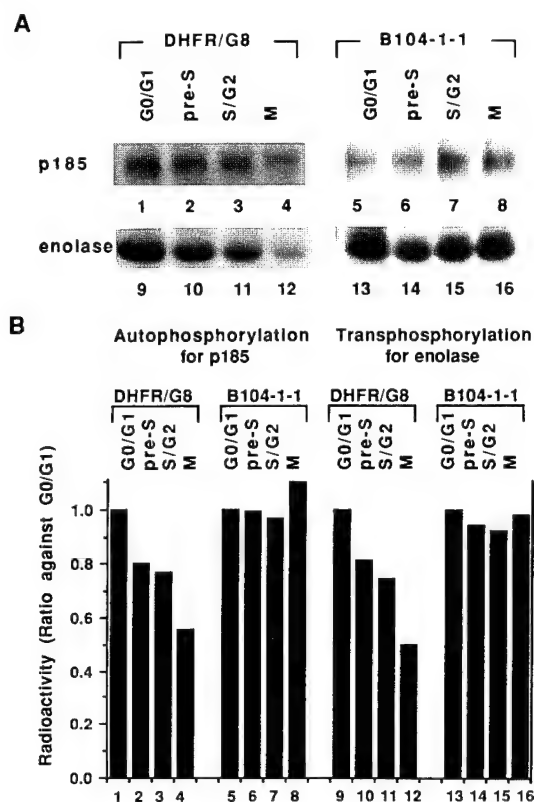


FIG. 3. Intrinsic kinase activity of p185^{neu} and p185^{neu*} in each cell cycle stage. (A) Enzymatic activities of p185^{neu} and p185^{neu*} in different cell cycle stages were tested by an *in vitro* immune complex kinase assay. After immunoprecipitation from 500 μ g of each cell sample with monoclonal anti-p185^{neu} antibody c-neu(Ab-4) (Oncogene Science), p185^{neu} or p185^{neu*} protein from each cell cycle stage was incubated for 20 min at room temperature with both enolase, as an exogenous substrate for p185^{neu}, and [γ -³²P]ATP in kinase buffer. After separation on a SDS/7% polyacrylamide gel, samples were exposed on x-ray film. To confirm that the protein level of p185^{neu}/p185^{neu*} in each lane was comparable, immunoprecipitation was carried out in duplicate and examined by immunoblotting with monoclonal anti-p185^{neu} antibody c-neu(Ab3) (data not shown). Lanes: 1–8, autophosphorylation for p185^{neu}/p185^{neu*}; 9–16, transphosphorylation for enolase; 1–4 and 9–12, DHFR/G8 cells; 5–8 and 13–16, B104-1-1 cells. The reproducible results were obtained from three independent experiments. A typical result is shown. (B) Radioactivity of each p185^{neu}/p185^{neu*} (columns 1–8) or enolase (columns 9–16) was measured on a Betascope 603 (Betagen, Waltham, MA) and is indicated as a ratio against the radioactivity in G₀/G₁ phase. Average from three independent experiments is presented as a graph. Columns 1–4 and 9–12, DHFR/G8 cells; columns 5–8 and 13–16, B104-1-1 cells.

varies in the different cell cycle stages *in vivo*, we analyzed the tyrosine phosphorylation state of p185^{neu} and its substrates in DHFR/G8 cells from different cell cycle stages by immunoblotting with anti-phosphotyrosine [Tyr(P)] antibody. As shown in Fig. 4A, p185^{neu} showed different levels of Tyr(P) content in a cell cycle-dependent manner. The Tyr(P) content of p185^{neu} was highest in G₀/G₁ phase and lowest in M phase. We also observed phosphorylated proteins with molecular masses of 150 and 120 kDa (noted as p150 or p120, respectively, in Fig. 4A). Interestingly, the profile of Tyr(P) content in these proteins was similar to p185^{neu}. However, in B104-1-1 cells, p185^{neu*}, p150, and p120 showed a high Tyr(P) content throughout the cell cycle. It has previously been shown that p185^{neu} has several substrates including PLC- γ (150 kDa) and GAP (120 kDa), which can bind to p185^{neu} (8). Since, by immunoblotting analysis, we found that both p150 and p120 were able to be coimmunoprecipitated by an anti-p185^{neu} antibody indicating their physical association with p185^{neu}

(Fig. 4B), and migration rate of p150 and PLC- γ or p120 and GAP on SDS/PAGE were identical (data not shown), it is likely that p150 and p120 may represent PLC- γ and GAP, respectively. To confirm this, we further demonstrated that Tyr(P) levels of PLC- γ and GAP were regulated in a similar cell cycle-dependent manner (Fig. 4C). We immunoprecipitated PLC- γ protein from both cell lines in G₀/G₁ and M phases with anti-PLC- γ antiserum and examined the proteins by immunoblotting with anti-Tyr(P) antibody and anti-PLC- γ antibody. As shown in Fig. 4C, while the protein levels of PLC- γ measured by anti-PLC- γ antibody remain identical between G₀/G₁ and M phases, their profile of Tyr(P) content was coincident with that of p150 in Fig. 4A. The Tyr(P) level of PLC- γ is much lower in M phase than in G₀/G₁ phase for DHFR/G8 cells. However, in B104-1-1 cells, the Tyr(P) level of PLC- γ is independent of the cell cycle. Similar results were found for GAP (Fig. 4C). These results indicate that the function of p185^{neu}, including both autophosphorylation for p185^{neu} and transphosphorylation for its substrates *in vivo*, is regulated in a cell cycle-dependent manner and is least active at M phase. However, transforming p185^{neu*} has escaped this cell cycle regulation and remains active through the entire cell cycle.

Altered Phosphorylation Is the Major Factor for Cell Cycle-Dependent Regulation of p185^{neu}. We next examined the mechanism of cell cycle-dependent regulation of p185^{neu}. It has been shown that several kinds of proteins reveal altered electrophoretic mobility on SDS/PAGE in a cell cycle-specific manner (19, 21, 23). In most cases, increased phosphorylation of serine and/or threonine residues accounts for a retarded electrophoretic mobility (19, 21). Therefore, we attempted to determine whether the varying migration of p185^{neu} throughout the cell cycle might also be caused by altered phosphorylation of serine and/or threonine residues. By treating DHFR/G8 and B104-1-1 cells arrested in G₀/G₁ and M phases with calf intestinal alkaline phosphatase, we showed that p185^{neu} from DHFR/G8 cells in M phase has the same mobility as in G₀/G₁ phase (Fig. 5). Since p185^{neu} contains much fewer phosphorylated tyrosine residues in M phase (Fig. 3A), these results suggest that the migration shift of p185^{neu} in M phase is mainly caused by phosphorylation of serine and/or threonine residues. In the case of B104-1-1 cells, the high molecular weight form of p185^{neu*} was also caused by phosphorylation, as it was no longer present throughout the cell cycle after calf intestinal alkaline phosphatase treatment (data not shown).

DISCUSSION

Our data demonstrate that the structure and function of p185^{neu} are regulated through the cell cycle. In M phase, p185^{neu} is least active in tyrosine phosphorylation function. Cell cycle regulation of p185^{neu} may occur through the alteration of phosphorylation of serine and/or threonine residues. It has been shown that nonreceptor type tyrosine kinases such as Src and Abl are also regulated during the cell cycle. For example, phosphorylation state of Src is regulated in a cell cycle-specific manner and its activity can be activated during G₁ phase after stimulation with certain growth factors (19, 20, 29). In contrast to p185^{neu} (Fig. 3), the mitosis-specific Src has enhanced tyrosine transphosphorylation activity on enolase (19). In the case of c-Abl, its binding activity to the DNA is regulated by cdc2-mediated phosphorylation, and its function is also regulated through a specific interaction with the retinoblastoma protein during the cell cycle (21, 22). Specific serine/threonine residues in Src and Abl have been identified and shown to be substrates of cdc2 kinase. Similar to p185^{neu}, the cell cycle-dependent serine/threonine phosphorylation of Src and Abl also showed retarded migration in M phase. Furthermore, the differential phosphorylation of c-Abl in the cell cycle is determined by an equilibrium between cdc2 kinase and protein phosphatase activities. The cdc2, a serine/

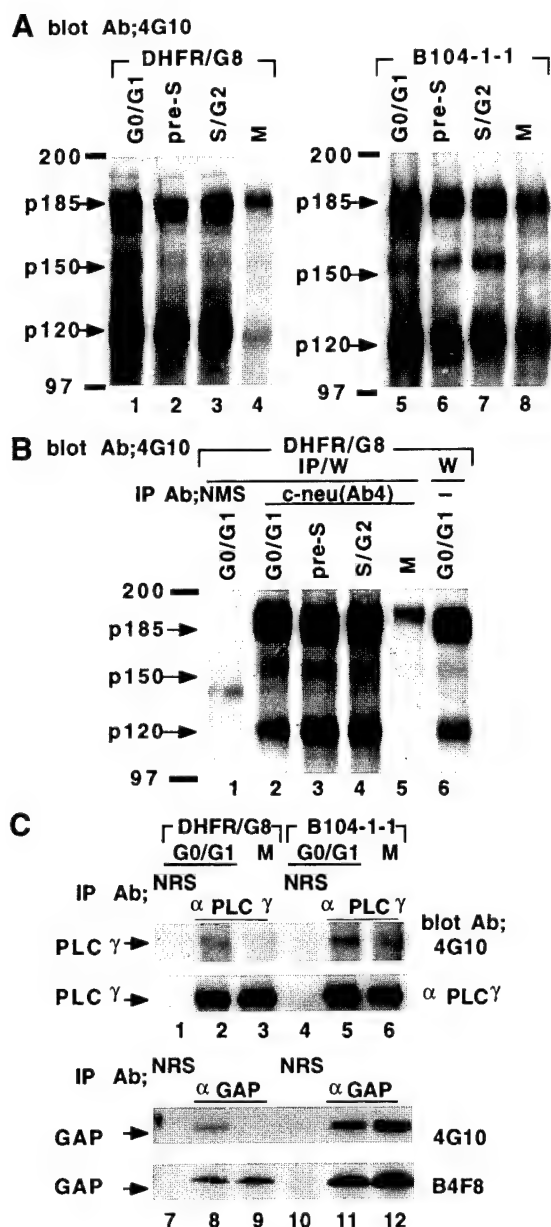


FIG. 4. Cell cycle-dependent tyrosine phosphorylation of p185^{neu} and its substrates in DHFR/G8 cells but not p185^{neu} in B104-1-1 cells. (A) Cell lysates (50 μ g per lane) from both DHFR/G8 cells (lanes 1–4) and B104-1-1 cells (lanes 5–8) in each cell cycle stage were separated on a SDS/6% polyacrylamide gel and examined by immunoblotting with monoclonal anti-Tyr(P) antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) under the same experimental conditions as described in Figs. 1B and 2B. In addition to p185^{neu}, two major phosphorylated proteins, which were found on this analysis, are indicated as p150 and p120 according to their molecular mass. (B) Immunoprecipitated p185^{neu} and their associated proteins from DHFR/G8 cells were tested by immunoblotting with monoclonal anti-Tyr(P) antibody. Proteins were immunoprecipitated with either monoclonal anti-p185^{neu} antibody c-neu(Ab-4) (lanes 2–5) or normal mouse serum (NMS; Pierce) (lane 1) from 500 μ g of each cell extract as indicated. After intensive washing with lysis buffer, samples were separated on a SDS/6% polyacrylamide gel and immunoblotted with monoclonal anti-Tyr(P) antibody 4G10. As a control, 50 μ g of G₀/G₁-phase cell extracts were loaded on the same gel (W/-; lane 6). (C) Tyr(P) contents of immunoprecipitated PLC- γ protein and GAP were tested by immunoblotting. Proteins were immunoprecipitated with either anti-PLC- γ antiserum (α PLC- γ ; Upstate Biotechnology) (lanes 2, 3, 5, and 6) or normal rabbit serum (NRS; Pierce) (lanes 1 and 4) from 500 μ g of cell extracts from both DHFR/G8 cells (lanes 1–3) and B104-1-1 cells (lanes 4–6) in either G₀/G₁ or M phase. After dividing the samples into two equal parts, immunoprecipitates were



FIG. 5. Effect of alkaline phosphatase treatment on mobility of p185^{neu}. p185^{neu} proteins were immunoprecipitated from 100 μ g of cell lysates from DHFR/G8 cells arrested in either G₀/G₁ or M phase. After treatment with (lanes 3 and 4) or without (lanes 1 and 2) 20 units of calf intestinal phosphatase (CIP; New England Biolabs), samples were separated on a SDS/6% polyacrylamide gel and subjected to immunoblot analysis with monoclonal anti-p185^{neu} antibody c-neu(Ab3). Numbers on left are kDa.

threonine kinase, is a key regulator of several growth-related proteins and is most active in M phase (20, 25, 30, 31). The amino acid consensus recognition sequence for serine and threonine phosphorylation sites has been proposed to be Ser/Thr-Pro, especially when included within larger sequences of either basic-Ser/Thr-Pro-polar-basic or basic/polar-Ser/Thr-Pro-Xaa-basic (20, 24, 32). Since our data suggest that p185^{neu} is hyperphosphorylated on serine and/or threonine residues in M phase, the amino acid sequence of p185^{neu} was examined and 16 consensus Ser/Thr-Pro sites, including one basic-Ser-Pro-polar-basic amino acid sequence, were found in p185^{neu}. Our preliminary results also suggest that cdc2 might be associated with p185^{neu} (unpublished observation); however, more rigorous experiments need to be done to confirm its physiological significance. If this turns out to be true, tyrosine kinase receptors may be another class of cellular substrates regulated by the cdc2 kinase. It is certainly an interesting issue, and it remains to be seen whether tyrosine kinase receptors will be regulated in a similar way as nonreceptor tyrosine kinases by cdc2 kinase.

One very interesting result shown in this report is that mutated p185^{neu*} escaped from this cell cycle regulation. Thus, point mutation of p185^{neu*} (Val-664 to Glu) may disrupt cell cycle regulation and allow p185^{neu*} tyrosine kinase to be constitutively active throughout the cell cycle. This cell cycle-independent function provides an attractive interpretation for the previous observation that p185^{neu*} is associated with much higher overall tyrosine kinase activity than p185^{neu} (6, 7). Cell cycle-independent function may also define a specific mechanism for p185^{neu*}-mediated cellular transformation. It has previously been shown that p185^{neu*} predominantly exists as a dimer form and p185^{neu} exists as a monomer form (33). By analogy to epidermal growth factor receptor (34, 35), the dimer form is believed to be an active molecule, and an equilibrium between dimer and monomer of p185^{neu} controls

immunoblotted with either monoclonal anti-Tyr(P) antibody 4G10 or monoclonal anti-PLC- γ antibody (α PLC- γ ; Upstate Biotechnology). The same experiment was carried out for GAP by immunoprecipitating with either polyclonal anti-GAP antibody (α GAP; Upstate Biotechnology) (lanes 8, 9, 11, and 12) or normal rabbit serum (lanes 7 and 10) and then immunoblotting with monoclonal anti-Tyr(P) antibody 4G10 or monoclonal anti-GAP antibody B4F8 (Upstate Biotechnology).

activity of p185^{neu}. For example, upon interaction with a ligand, p185^{neu} may change its conformation to favor a dimer form, which enhances tyrosine kinase activity and therefore induces a signal transduction pathway. It would be interesting to see whether equilibrium between dimer and monomer forms of p185^{neu} is also regulated in a cell cycle dependent manner. If so, we predict that p185^{neu} should favor the dimer form in the G₀/G₁ phase and, through the progression of the cell cycle, the dimer form gradually decreased to the least amount in M phase. We also predict that p185^{neu} predominantly remains as a dimer form through the entire cell cycle. If this indeed is the case, hyperphosphorylation of p185^{neu} on serine/threonine residues in M phase may alter conformation of p185^{neu} to prevent dimer formation. Thus, studies on cell cycle-dependent serine/threonine phosphorylation may help to identify critical phosphorylation sites that control p185^{neu} conformation and regulate equilibrium between dimer and monomer forms of p185^{neu}.

We have also recently observed that epidermal growth factor receptor exhibits a similar cell cycle-dependent migration shift on SDS/PAGE and altered phosphorylation on tyrosine residues during the cell cycle (unpublished data). This cell cycle-dependent regulation may therefore be a common phenomenon of receptor tyrosine kinases. In summary, the study described in this report provides a view on regulation of receptor tyrosine kinases and may lead to understanding how cell cycle control regulates cellular transformation induced by tyrosine kinase receptor molecules.

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Regulation of HER2/*neu* gene expression (Review)

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Abstract. Transcriptional regulation of the HER2/*neu* proto-oncogene (also known as *c-erbB2*) has been the topic of many recent reports. The importance of these studies lies in the fact that HER2/*neu* overexpression occurs in about 30% of breast and ovarian cancers and to varying degrees in other cancers, including gastric, colorectal, lung, salivary, ovarian, bladder, pancreas, endometrial, cervical, oral, and prostate cancers. Furthermore, increased levels of the gene product play an important role during the neoplastic process of certain tumors. This review summarizes the recent work studying the regulation of HER2/*neu* expression in cells that overexpress the gene and reviews the current knowledge on general regulation of the HER2/*neu* promoter.

Contents

1. Introduction
2. Basic promoter structure
3. *cis*-acting regulatory elements
4. Overexpressing elements
5. Other regulators
6. Summary

1. Introduction

Amplification and/or overexpression of the HER2/*neu* proto-oncogene occur in approximately 30% of breast cancers (for a review see ref. 1). These phenomena have been associated with poor patient prognosis, including shorter time to relapse and shorter overall survival time (2). Since overexpression of the gene in the absence of gene amplification has been demonstrated in up to 26% of breast cancers, regulation of

HER2/*neu* expression is an important area of study. As an indication of what might be occurring *in vivo*, several groups have used established cell lines as model systems. Many groups have been examining the breast cancer cell lines first identified by Kraus and colleagues as cells that overexpress HER2/*neu* to a significant degree over the level of gene amplification (3). Gene expression can be deregulated by several different mechanisms. In broad terms, increased mRNA levels in the absence of gene amplification could be caused by either increased transcription or deregulation at the post-transcriptional level. A post-transcriptional deregulation event that leads to an increased steady-state level of mRNA could be an increase in the stability of the mRNA, an alteration in splicing rate, a change in the nuclear-to-cytoplasm transport rate, a change in the 5' capping rate, or a change in the poly-A tail addition rate. Three groups that have examined post-transcriptional deregulation of the HER2/*neu* gene looked only for a change in the mRNA half-life and did not detect an increase in cells that overexpress HER2/*neu* (4-6). Thus, transcriptional deregulation has been the primary focus of researchers attempting to define the mechanism(s) by which overexpression of HER2/*neu* in breast cancer occurs.

Increased transcription can occur by either deregulation in a *cis*-acting DNA element or a trans-acting factor. This deregulation can result in either an increased association of a positive-acting factor or a decreased association of a negative factor and may be caused by increased levels or affinity of a positive factor or decreased levels or affinity of a negative factor (Fig. 1). Much of what we know about HER2/*neu* transcriptional control was determined by asking if known trans-acting factors, e.g., *c-myc*, estrogen receptor (ER), can regulate the promoter. The other common approach was to examine cells that already overexpressed the gene and find out what was causing the overexpression. The cloning of three species' promoters (mouse, rat, and human) has prompted the identification of several DNA elements and trans-acting factors that regulate expression of HER2/*neu*. Because some factors (e.g., *c-myc*) are apparently not functional in all species and in all cell types, understanding the regulation has been difficult. Furthermore, the use of different cell lines has generated data that does not always coincide, making interpretation sometimes difficult.

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Abbreviations: ER, estrogen receptor; CAT, chloramphenicol acetyl transferase; EGF, EGFR, epidermal growth factor and its receptor; tk, thymidine kinase; Rb, retinoblastoma

Key words: HER2/*neu*, oncogene

2. Basic promoter structure

Our laboratory has conducted a series of experiments to map *cis*-acting promoter elements and to identify the trans-acting

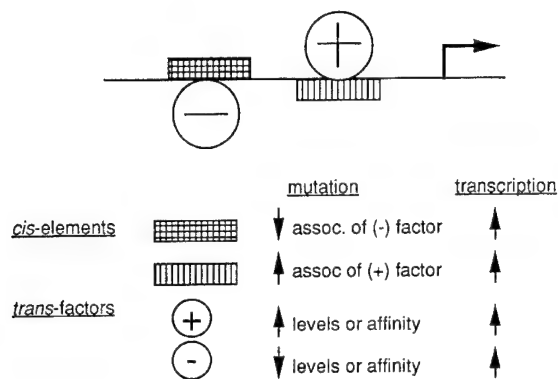


Figure 1. Model for increased transcription of a gene. Certain mutations in DNA elements (indicated by boxes) or transcription factors (indicated by circles) will result in an overall increase in gene transcription.

factors that interact with these sequences. Using a 2.4-kb fragment of the rat *neu* promoter and a series of 5'-deletions to drive expression of the chloramphenicol acetyl transferase (CAT) gene, three positive-acting *cis* elements and one negative-acting *cis* element were identified (7). A 649-bp sequence of the rat *neu* promoter stretching from -504 to +145 relative to the translation start site was sequenced and characterized. The promoter contains a CAAT box, lacks a TATA box, and is 75% G-C rich. Four transcription start sites were identified at -143, -147, -158, and -203. Subsequent cloning of the mouse *neu* promoter by our laboratory revealed 93% homology with the rat *neu* promoter (8). The mouse promoter is also G-C rich (67%), contains a CAAT box, and lacks a TATA box. Cloning of the human HER2/*neu* promoter was accomplished by three separate groups (9-11). The human promoter is 78% homologous to

the mouse *neu* promoter (8) and has at least one major and two minor transcription initiation sites at -178, -244, and -257 (9) or could have as many as seven initiation sites (10). The human HER2/*neu* promoter also contains a CAAT box; however, unlike the mouse and rat promoters, the human promoter has a TATA box as well. Several features are shared by all three species' promoters. All have at least one Sp1 binding site, an OTF-1 binding motif, an RVF binding site (see below), two AP2 consensus sequences, at least one E4TF1 binding site, a PEA3 consensus site, and several GGA repeats. In addition, the rodent promoters have GCAA repetitive motifs (4 in mouse, 13 in rat), and a GCF binding site neither of which are present in the human promoter.

When a 1538-bp region of the human HER2/*neu* promoter was used to drive expression of the luciferase gene, the promoter was determined to have an equivalent amount of activity as the epidermal growth factor receptor (EGFr) promoter (11). The promoter is 32% as active as the Herpes simplex thymidine kinase (tk) promoter and 16% as active as the Rous sarcoma virus promoter. In addition, activity of the human promoter could be induced by EGF, TPA (12-O-tetradecanoylphorbol-13-acetate), dibutyryl cAMP, and retinoic acid. Treatment with combinations of these inducing agents resulted in additive and synergistic responses, suggesting complex regulation of the HER2/*neu* promoter (11).

3. *cis*-acting regulatory elements

The largest 5' regulatory region of HER2/*neu*, cloned by Grooteclaes and colleagues, extends to a point 6.2-kb upstream of the translation initiation site (12; Fig. 2). The region between -6.2 kb and -4 kb functions as an activator of transcription in BT474 breast cancer cells but not in the HBL100, T47D, and MDA-MB453 breast cell lines (12; Table I). Deletion of this promoter region in BT474 cells

Table I. Cell lines.

Cell line	Type	HER2/ <i>neu</i>	
		mRNA level	gene amplification
Human			
HeLa	cervical carcinoma	1	1
HBL100	normal breast	1	1
T47D	breast carcinoma	1	1
MCF7	breast carcinoma	1	1
ZR75-1	breast carcinoma	8	1
MDA-MB453	breast carcinoma	64	2
BT474	breast carcinoma	128	4-8
BT483	breast carcinoma	8 (64)	1 (1-2)
Rodent			
Rat-1	immortalized	1	1
NIH3T3	immortalized	1	1
B104-1-1	transformed	10	10 (<i>neu</i> *)
DHFR-G8	transformed	50-100	100

*point-mutated transforming gene (27).

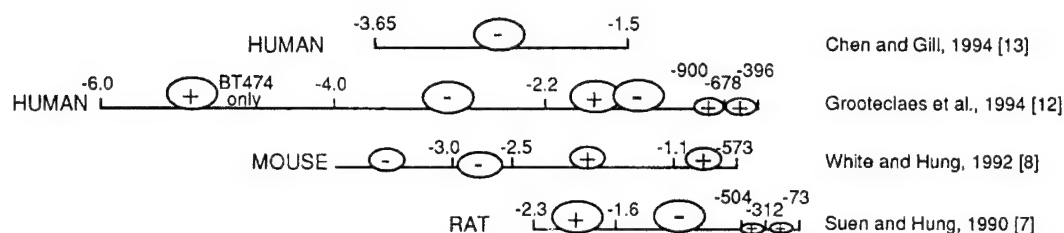


Figure 2. HER2/*neu* promoter elements. The net effect of various promoter regions on transcriptional activity as determined by different investigators are indicated. Numbers are approximate kb (1.1-6.0) or bp (73-900) distances upstream from the gene translation start codon.

results in a drop of transcriptional activity from 14 to 0.6 fold, compared with the smaller HER2/*neu* promoter, -396 to -138. Promoter activity in HeLa cells also decreases when this 2.2-kb fragment is deleted, albeit to a lesser extent, from 3.5 to 1.0 fold above basic promoter activity. The same researchers also identified the next promoter fragment between 4-kb and 2.2-kb upstream of the initiation codon to function as a repressor of transcription. Insertion of this fragment upstream of the tk minimal promoter conferred transcriptional repression up to 70% in HeLa cells. However, in the reverse orientation, no effect was observed, suggesting that this element does not behave as a classical silencer. Chen and Gill (13) found an overlapping region from -3.65 to -1.5-kb to contain repressor activity in HeLa and CV-1 cells (Fig. 2). In work by our group using the mouse promoter, the region between -3.0 and 2.5-kb upstream of the translation start site was shown to function as a silencer and to repress the tk promoter by 40% in NIH3T3 cells and 70% in Rat-1 cells (8). Using DNase I footprinting and methylation interference techniques, two protein binding sites within this 568-bp region were identified. Each of these individually cloned back into the mouse promoter reduces promoter activity by up to 51%, one site working best in the 5'-3' orientation and the other working best in the reverse orientation, suggesting positional effects and perhaps cooperation between the two sites (White-Jones, Miller, and Hung, in preparation).

The region between -2.2 and -0.9-kb in the human promoter has different activities, depending on the cell line examined (12). Deletion of this region results in a drop of promoter activity in HBL100 cells suggesting the presence of a transcriptional enhancer. Deletion in HeLa cells has no effect on promoter activity; however, deletion results in increased promoter activity in T47D, MDA-MB453, and BT474 cells, suggesting the presence of a silencer. The different effects observed in the different cell lines likely reflects the presence and/or absence of nuclear factors in different cell lines that can regulate HER2/*neu* transcription.

In the rat promoter, this region was examined in two different parts: -2.3 to -1.6-kb and -1.6 to -0.5-kb. The upstream 0.7-kb acts as an enhancer in Rat-1 fibroblasts, because deletion of this region results in 50% of the initial activity, and cotransfection with increasing amounts of this promoter fragment reduces promoter activity to below 15%. The downstream 1.1-kb acts as a silencer, repressing activity

from 260% to 50% above minimal promoter activity (7). When similar domains were examined in the mouse promoter, the upstream region from -2.5 to -1.1-kb contained weak enhancer activity, whereas the region between -1.1-kb and -573 bp had strong enhancer activity (8; Fig. 2).

The apparent discrepancies among promoters is likely caused by a difference in sequences. The region upstream of -500 bp has not been sequenced in the rat HER2/*neu* promoter. Certainly, the high degree of homology (>78%) between rat, human, and mouse promoters in the region closest to the start of translation could deviate as more 5' regions are examined. Furthermore, because true silencers and enhancers function independently of their distance from the transcription start sites, a similar silencer or enhancer, responding to similar cellular signals and interacting with similar protein factors, may be at one location in one species' promoter but at a different location in another species' promoter.

An enhancer was also noted between -900 and -678 bp in the human promoter (12); however, this was most active in BT474 cells, conveying 9-fold higher promoter activity compared with its absence. This element increased promoter activity in the other human lines tested by an average of 2.5-fold.

4. Overexpressing elements

To identify the mechanism for increased HER2/*neu* mRNA levels in breast cancer cells, one must first determine by nuclear run-on analysis if the increase in mRNA is caused by an increase in the transcription rate. If, indeed, the HER2/*neu* transcription rate is higher in a particular line, then one can test for an alteration in *cis* or in *trans* by performing transient transfections with the promoter linked to a reporter gene. Cells that contain an altered factor that acts in *trans* upon the HER2/*neu* promoter will have higher reporter activity, whereas cells with an alteration in a *cis*-acting sequence will not. Alternatively, cells lacking higher promoter activity might still contain an altered *trans*-acting factor if the region with which it interacts is not present in the promoter-reporter construct or if its quantities are limited such that an abundance of reporter constructs would saturate its supply.

ZR75-1 human breast cancer cells were demonstrated by Hollywood and Hurst to have a 3.8-fold increase in HER2/*neu* transcription compared with T47D breast cancer

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-677 CCCGGGGGTC CTGGAAGCCA CAAGGTAAAC ACAACACATC CCCCTCCTTG ACTATGCAAT

-617 TTTACTAGAG GATGTGGTGG GAAAACCATT ATTTGATATT AAAACAAATA GGCTTGGGAT

-557 GGAGTAGGAT GCAAGCTCCC CAGGAAAGTT TAAGATAAAA CCTGAGACTT AAAAGGGTGT
                                RVF factor

-497 TAAGAGTGGC AGCCTAGGGA ATTTATCCCG GACTCCGGGG GAGGGGGCAG AGTCACCAGC

-437 CTCTGCATTT AGGGATTCTC CGAGGAAAAG TGTGAGAACG GCTGCAGGCA ACCCAGGCGT
                                PstI/OB2-1
                                StuI
-377 CCCGGCGCTA GGAGGGACGA CCCAGGCCTG CGCGAAGAGA GGGAGAAAGT GAAGctggga
                                BCREG                               Sp1

-317 gttgccgact cccagACTTC GTTGGAATGC AGTTggaggg ggcgagctGG GAGCGCGCTT
                                E1A                               GTG/Sp1                               Pal
                                XhoI
-257 GCTCCCAATC ACAGGagaag gaggaggTGG AGGAGGAGGG CTGCTTGAGG AAGTATAAGA
                                Sp1                               RBII                               TATA

-197 ATGAAGTTGT GAAGCTGAGA TTCCCCTCCA TTGGGACCGG AGAAACCAGG GGAGCCCCC
                                +1

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Figure 3. Sequence of human HER2/*neu* promoter (9,11). Arrowheads indicate StuI and XhoI restriction points, although the recognition site for XhoI is only present in the mouse and rat promoters. Sp1 sites as identified by Chen and Gill (13) are in lower case letters. Trans-factor responsive elements are underlined and labeled. Line numbers are relative to the translation start site (+1).

cells that do not overexpress the gene (4). Furthermore, a HER2/*neu* promoter-driven CAT reporter gene was 6-fold more active in ZR75-1 cells than in T47D cells. In an attempt to identify the cause of increased HER2/*neu* mRNA in ZR75-1 cells, a region between -396 and -390 in the human promoter was identified as a protein binding site by DNase I footprinting. This sequence was only bound in the overexpressing lines ZR75-1 and BT483 and not in the underexpressing lines HBL100 and T47D. When an oligonucleotide containing this region was used in gel shift analysis, the protein complex formed was more abundant in six HER2/*neu*-overexpressing breast cancer cell lines compared with normal breast epithelial cells, HBL100. When cloned in triplicate upstream of a basal promoter, this region directed a 6-fold increase in activity in ZR75-1 cells and not in T47D cells. The trans-acting factor that interacts with this promoter region was termed OB2-1 (overexpression of *c-erbB2*, factor 1) (4). These results were not observed by other groups, probably because the OB2-1 core binding site contains the recognition sequence for PstI and was thus disrupted in 5' serial deletions of the promoter using restriction enzymes. The OB2-1 binding core sequence is not conserved in the mouse and rat promoters, suggesting that its functional significance may not extend to rodents.

The undertaking of a similar study by our group identified BT474, BT483, and MDA-MB453 breast cancer cells as lines that have increased transcription of the HER2/*neu* gene. Of these only MDA-MB453 had higher promoter activity in transient transfections with a HER2/*neu* promoter-driven reporter gene, suggesting the presence of an altered factor that acts in trans upon the promoter (5). Initial experiments

using serially-deleted rat promoter plasmids indicated a 13-bp DNA element between -312 and -299 in the rat promoter to be required for transcriptional trans-activation in MDA-MB453 cells. An oligonucleotide containing this region could interact in a DNA-specific manner with nuclear extracts from these cells and could confer the promoter partially responsive to trans-activation by MDA-MB453. Using serially deleted human promoter constructs in these cells, the same but overlapping promoter region was determined to be critical to transcriptional trans-activation in MDA-MB453 cells (Miller and Hung, in preparation). This region, named BCREG (breast cancer regulator), was also able to complex with nuclear factors from the breast cancer cells and could increase basal HER2/*neu* promoter activity by 4-5 fold in overexpressing MDA-MB453 cells but not in control MCF-7 cells. The DNA nucleotides implicated in protein contact by methylation interference experiments are 100% conserved across rat, human, and mouse. UV-crosslinking studies suggest that polypeptides of approximately 110, 70, and 35 kDa are capable of complexing with this DNA element.

In work by Sarkar and colleagues, the transcriptional initiation site was demonstrated to be critical to HER2/*neu* overexpression (14). Using the 31-bp DNA sequence flanking the major transcription initiation site in the human HER2/*neu* promoter (-199 to -166 relative to the translation start site) in gel shift analysis indicated that cytoplasmic extract from malignant breast cancer tissue contained factors that could interact with this sequence, whereas extract from normal breast tissue did not. Binding activity was also found, albeit to a lesser extent, in malignant tissue from lung,

kidney, and pancreas. Southwestern analysis indicated that this factor is approximately 45-48 kDa in size. The authors term this factor HPBF (HER2/*neu* promoter binding factor). Following affinity column purification and injection into serum-starved NIH3T3 cells, this factor could induce surface expression of p185HER2/*neu* and moderate levels of DNA synthesis (14). Although malignancy correlated with increased levels of HPBF, a correlation with increased transcription of the HER2/*neu* gene has not yet been shown. The possibility that HPBF may bind to the transcription initiation sites of other genes and perhaps induce expression of key proteins involved in malignancy is not yet clear. Although this region is 84% conserved in the rodent promoters, a transcription initiation site at an analogous position was not detected in the rat promoter (7). The mouse transcription initiation sites have not yet been mapped.

5. Other regulators

RVF. A positive *cis*-acting element was localized in the -504 to -312 region of the rat promoter and can increase promoter activity by 2.9 fold in Rat-1 cells (7; Fig. 2). Deletion of this region resulted in a loss of 38% of the promoter activity (15). The region between -678 and -396 of the human promoter likewise acts as a transcriptional enhancer (up to 3.3-fold) in five human cell lines (12). Using gel shift analysis and methylation interference, our group identified a promoter sequence, AAGATAAAACC (Fig. 3; -466 to -456 in the rat promoter), that binds a specific trans-acting factor termed RVF (EcoRV factor) (15). Extracts from both mouse (SW3T3) and human (HeLa) cells contain this binding activity. The RVF recognition site is 100% conserved in mouse, human, and rat promoters and can enhance promoter activity in any of these three species when cloned upstream of the minimal tk promoter in either orientation, indicating it to be a classical enhancer. Southwestern analysis demonstrated that a polypeptide of approximately 60 kDa in SW3T3 cells can bind to the RVF site.

Sp1. Sites that match the Sp1 transcription factor consensus have been identified in the rat, mouse, and human HER2/*neu* promoters (Fig. 3). One of these sites in the -323 to -304 region identified by Chen and Gill (13) is also a palindromic sequence and is 86% homologous among the rat, mouse, and human promoters. This sequence lies within a region identified as an enhancer (-329 to -230). Deletion of this enhancer region results in an overall 28-fold drop in promoter activity in HeLa and CV-1 cells. Cotransfection of Sp1 expression plasmids with the HER2/*neu* core promoter (-323 to -137) increased promoter activity by 28-fold in the same cell types; however, this Sp1 site does not provide most of the trans-activation activity in this region, because deletion of the palindrome/Sp1 recognition sequence results in only a 3-fold drop in activity. Two other Sp1 sites were identified in the human promoter but are apparently not as functionally important, because deletion of these sites did not affect the ability of cotransfected Sp1 to activate the promoter once the first Sp1 site had already been deleted (13). All three sites were identified by DNase I footprinting with Sp1 protein. A region overlapping with the latter two sites was also

identified as a protein binding site by Hollywood and Hurst who used breast cancer cell extract to perform DNase I footprinting (4). A second palindromic sequence overlapping the CAAT box at position -270 to -251 can, in triplicate, increase the tk promoter activity by 22-fold (13).

E1A and SV40 LT. While searching for transcriptional factors that may regulate HER-2/*neu* transcription, our group identified E1A of adenovirus as a transcriptional repressor of the HER-2/*neu* promoter (16). We found that E1A could repress HER2/*neu* transcription and that this transcriptional effect required the presence of a 139-bp StuI-XhoI promoter fragment (-312 to -173 in the rat promoter) (16; Fig. 3). Within this DNA region lies a consensus E1A responsive element, TGGAATG, at position -260 to -254 in the rat promoter, which is 100% conserved in the mouse and human promoters. At about the same time, an independent study from our group used adenovirus mutants to demonstrate that E1A protein could mediate downregulation of the HER-2/*neu*-encoded p185 protein (17). This study was based on an existing hypothesis from Carlin *et al* (18). In their study, Carlin and colleagues showed that the 10.4 kDa polypeptide encoded by the adenovirus E3 region induced downregulation of EGFR through endocytosis after adenovirus infection and hypothesized that the 10.4 kDa polypeptide may also downregulate p185 by a similar mechanism. While we tested this hypothesis, we found adenovirus could indeed downregulate p185 but that E1A and not E3 is required for the downregulation of p185 (17). Another viral oncoprotein, the simian virus 40 large T antigen (LT) was also shown to repress HER2/*neu* promoter activity (19). LT shares structural and functional homology to E1A; however, LT represses the HER2/*neu* promoter via a different mechanism than E1A, because the region of the HER2/*neu* promoter mediating repression by LT (-172 to -79) is downstream from the region responding to E1A (-312 to -173).

GTG enhancer. When the effect of the HER2/*neu* gene product on its own promoter was examined by our group, the gene was found to transcriptionally downregulate its own expression (20). Again, the StuI-XhoI region of the rat promoter was the element identified to mediate this phenomenon. Gel shift assays and methylation interference experiments demonstrated that a 10-bp element, GGTGGGGGGG, at position -243 to -234 in the rat promoter interacts with specific protein complexes. This element was shown to be sufficient to mediate HER2/*neu* autoregulation when cloned adjacent to the tk promoter. Because even in the absence of cotransfected HER2/*neu* expression plasmid this element could stimulate promoter activity by approximately 10-fold in either orientation, the element was classified as an enhancer. Interestingly, the point-mutated, constitutively active HER2/*neu* oncogene was a stronger repressor than wild-type proto-oncogenic HER2/*neu* (20). Although the GTG enhancer is also present at an analogous location in the mouse promoter, it is not conserved in the human HER2/*neu* promoter; however, the corresponding region in the human promoter, which retains five of seven of the downstream guanines, was found to be protected by DNase I footprinting using either Sp1 (13) or

cellular extract (4), suggesting that a trans-acting factor binding site may be present.

c-myc, *ER* and *Rb*. Our group also identified the StuI-XhoI (-312 to -173) region of the rat HER2/neu promoter to mediate downregulation by three other factors, the *c-myc* proto-oncogene, the retinoblastoma (Rb) tumor suppressor protein, and estrogen-stimulated ER. Mouse and human *c-myc* expression plasmids were stably transfected into B104-1-1 and DHFR-G8 cells, which overexpress the point-mutated, activated, and normal p185^{HER2/neu} proteins, respectively (21). *c-myc* was able to reverse HER2/neu-induced transformed morphology and downregulate the mRNA levels of HER2/neu in these cells. To determine if *c-myc* could regulate the HER2/neu promoter, transient transfections were performed using the serially-deleted rat HER2/neu promoter constructs in NIH3T3 cells. *c-myc* was found to inhibit the promoter up to 70%, and this effect required the presence of the StuI-XhoI region. In other work by our group, human *c-myc* was unable to inhibit the human HER2/neu promoter (22). This work was carried out in an *in vivo* system using inducible *c-myc* vectors in stably transfected human breast cancer cell lines that overexpress HER2/neu. Transient assays were not tested in these cells. The apparent discrepancy could be due to a difference in a species-specific factor since rodent cells were used in one study and human cells were used in the other or they could be due to a difference between the human HER2/neu and the rat HER2/neu regulatory sequences.

Estrogen-stimulated ER was also demonstrated to repress HER2/neu gene transcription via the StuI-XhoI promoter region (23). The responsive region in the human promoter was localized to the -396 to -139 region, which includes the analogous StuI-XhoI region. The pattern of nuclear factors binding to this region is altered in response to estrogen-stimulated ER, although the identity of these factors is not yet known. These results lend support to the idea that there is a causal relationship between loss of ER and overexpression of HER2/neu in primary breast carcinomas.

The retinoblastoma (Rb) susceptibility gene can also repress the activity of the rat HER2/neu promoter through the GTG enhancer element, because the presence of this element upstream of the tk promoter can mediate Rb-specific transcriptional downregulation in NIH3T3 cells (24). However, by using serially-deleted promoter fragments we found that the minimal 93-bp promoter (-172 to -79 in rat) was also repressed 70% by Rb (25). Fine deletion mapping of this region demonstrated that an 8-bp element, TCGAGGAA, was required for both efficient transcription and Rb-mediated downregulation. Interestingly, Rb apparently represses the basal promoter and the GTG element by different mechanisms since the SV40 large T antigen/E1A-binding domain of the Rb protein is required for repression via the GTG enhancer but is not required for repression of the basal promoter. Since the 8-bp element (-172 to -165) is only 6-bp upstream from one of the transcription initiation sites, Rb and this DNA sequence might interact with factors that are involved in transcriptional initiation. Although the GTG enhancer element is not conserved in the human HER2/neu promoter, this 8-bp element is 100% conserved in rat, mouse,

and human promoters. Nevertheless, Rb is nearly as effective in downregulating the human promoter (50-65%) as it is in repressing the rat promoter (60-80%) (25). It is interesting to note that transcriptional regulation of HER2/neu by Rb is cell type specific (26). Unlike its effects in NIH3T3 cells, Rb can not repress HER2/neu promoter activity in Rat-1 cells and can functionally antagonize transcriptional repression of HER2/neu by the adenovirus E1A protein. Therefore, Rb can regulate transcription of HER2/neu in a negative or positive manner, depending on the cell type.

6. Summary

In summary, expression of the HER2/neu gene is controlled by several negative-acting factors and several positive-acting factors. The actual levels of HER2/neu mRNA in a cell are probably regulated by combinations of these factors whose activities and/or levels likely change in response to extracellular signals via signal transduction pathways. As we have described, factors known to have a negative effect on HER2/neu transcription include the p185^{HER2/neu} protein, Rb, E1A, LT, ER, and *c-myc*, whereas factors functioning to increase promoter activity include EGF, TPA, dibutyryl cAMP, retinoic acid, and unidentified proteins binding to: OB2-1, RVF, BCREG, and the transcription initiation site.

The current experimental approaches to studying the activity of the HER2/neu promoter have a number of flaws. Since expression of the endogenous HER2/neu gene depends on *cis*-acting DNA sequences, then the longer promoter constructs used in *in vitro* systems and transient assays are more comparable with wild-type activity. However, one could imagine *cis*-acting sequences many kilobases from the gene promoter that have strong effects on gene expression. Thus, studies such as those described herein will disregard the effects of such elements if they exist. Furthermore, using small isolated promoter regions eliminates the effects of both upstream DNA elements as well as downstream. Many researchers clone *cis*-acting elements in tandem repeats to study their contribution to promoter activity. For example, Hollywood and Hurst used a plasmid containing three copies of the OB2-1 element upstream of a minimal promoter (4). Whether or not these types of arrangements are a reflection of what occurs *in vivo* is not clear. Furthermore, different groups have used different basal promoters, e.g., tk versus glutathione transferase π in conjunction with the DNA element under study. One must also keep in mind the standard to which the promoter is being compared. Our lab used a 2.2-kb rat HER2/neu promoter fragment as a standard in determining whether promoter regions were acting as enhancers or silencers (7). On the other hand, Grooteclaes and colleagues used their smallest promoter fragment (258 bp) as a standard (12). In addition, one cannot make conclusions about the activity of a certain promoter fragment without considering the cell line and, thus, the variance in trans-acting factors present. For example, HeLa cells, a cervix carcinoma cell line that does not overexpress the HER2/neu gene, cannot be compared with breast cancer cells that do not overexpress the gene (e.g., MCF7 and T47D) or likewise with breast cancer cells that do overexpress the gene (e.g., ZR75-1, BT474). As one can see from the existing data,

different promoter regions respond differently in various cell lines; likewise, cell lines may respond differently for different researchers. For example, work by Hollywood and Hurst did not detect changes in transcriptional ability in T47D cells as promoter regions were deleted (4); however, Grootclaes and colleagues detected variations to as much as 4.9-fold in these same cells when different promoter regions were examined (12). Similarly, our group measured the *in vitro* transcription rate of HER2/*neu* in BT474 cells as 5-fold higher than non-overexpressing cells, whereas Pasleau and colleagues measured it as 20 to 40-fold higher (6). Interestingly, the cells that we were using were ER⁻, whereas theirs were apparently ER⁺ (6,23); thus, heterogeneity within established cell lines must exist and may account for some of the discrepancies in these studies.

In conclusion, multiple mechanisms may be involved in HER-2/*neu* overexpression in breast cancer cells and transcriptional upregulation seems to be the most common mechanism identified among different breast cancer cell lines that overexpress HER-2/*neu*. Although further experiments are needed to clarify the interactions between HER2/*neu* promoter elements and the regulatory trans-acting factors among the different cell lines, the current studies suggest that identification of these factors and characterization of their responses to extracellular as well as intracellular signals will be potentially interesting. Certainly, these findings will help facilitate our understanding of how the expression of HER2/*neu* is deregulated in multiple human cancers.

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Signal Transduction of HER-2/neu Receptor Tyrosine Kinase

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The *neu* oncogene is a transforming gene originally isolated from an ethylnitrosourea-induced rat neuro/glioblastoma.¹⁻⁴ Its normal cellular counterpart, the normal *neu* gene, and the human homolog (named *HER-2* or *erbB2*) were isolated from rat and human libraries.^{5,6} It is now known that *neu*, *HER-2*, and *erbB2* are the same gene. We will use the term *HER-2/neu* in this review.

The *HER-2/neu* proto-oncogene encodes a 185-kd transmembrane glycoprotein (known as p185) with intrinsic tyrosine kinase activity homologous to the epidermal growth factor receptor (EGFR).^{3,7,8} Several lines of evidence have suggested a link between overexpression of this proto-oncogene and neoplastic transformation. First, overexpression of *HER-2/neu* induced transformation of NIH/3T3 cells⁹⁻¹¹ and mammary epithelial cells.¹² Second, transgenic mice overexpressing *HER-2/neu* developed mammary adenocarcinomas.¹³⁻¹⁵ Interestingly, many of the tumor-bearing transgenic mice developed secondary metastatic tumors in the lung.¹⁴ Third, amplification and/or overexpression of *HER-2/neu* have been detected in a variety of human carcinomas, including approximately 30% of breast and ovarian cancers.¹⁶⁻¹⁹ Aberrant expression of *HER-2/neu* is also detected in other human cancers, including gastric tumors^{20,21} and colon,^{22,23} lung,²⁴⁻²⁸

HER-2/neu (also called *erbB2*) encodes a 185-kd tyrosine kinase receptor, named p185, belonging to the class I receptor subfamily. Aberrant expression of *HER-2/neu* is implicated in many human cancers. An understanding of the signaling pathway of p185 may shed light on the mechanisms involved in p185-mediated neoplastic transformation or carcinogenesis. This article reviews recent work on the signal transduction of p185.

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oral,²⁹ cervical,³⁰ and prostate cancers.^{31,32} Moreover, amplification and overexpression of *HER-2/neu* have been associated with the presence of metastatic disease, increased probability of tumor recurrence, and poor patient survival.^{16,17,33-36} In addition to clinical reports, our laboratory has observed that enhanced expression of *HER-2/neu* increases malignancy, including the metastatic potential in mouse fibroblast cells,^{37,38} human lung cancer cells,³⁹ and human ovarian cancer cells.⁴⁰ These data are in good agreement with similar results from murine colon adenocarcinoma cells.⁴¹ Recently, overexpression of *HER-2/neu* has been shown to correlate with chemoresistance,⁴² compatible with the association between *HER-2/neu* overexpression and poor prognosis.

Although the oncogenic potential of *HER-2/neu* has been well documented, the molecular basis of *HER-2/neu*-induced transformation, specifically, the signal transduction

pathway of *HER-2/neu* is not well understood.

Activation of p185 Tyrosine Kinase

Polypeptide growth factors regulate cellular proliferation or differentiation by stimulating the intrinsic protein tyrosine kinase activities of their specific cell surface receptors. *HER-2/neu* is one member of the receptor tyrosine kinase subfamily (designated class I receptors⁴³) that has a characteristic distinct protein structure consisting of a cysteine-rich extracellular ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine kinase domain. Epidermal growth factor receptor is the prototype for this subfamily. In addition to EGFR and the *HER-2/neu*-encoded p185, 2 other family members, termed p180^{HER-3/erbB-3} and p180^{HER-4/erbB-4}, have recently been identified.^{44,45} Aberrant activation of the tyrosine kinase activities of these receptors has been proposed to contribute to tumorigenesis or progression.⁴⁶ Indeed, it has been demonstrated that tyrosine kinase is essential for the transforming ability of the *HER-2/neu* oncoprotein.^{47,48} Mutant p185 (Lys to Met at residue 757 in rat p185, Lys to Met at residue 753 in human p185) loses its ATP-binding site and tyrosine kinase activity and concurrently abolishes its transforming ability. Therefore, understanding the mechanisms by which p185 tyrosine kinase becomes activated is the first step to envisage the signal transduction pathway that mediates oncogenic processes.

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Point Mutation

Comparison of the transforming rat *HER-2/neu* oncogene sequence with its proto-oncogene counterpart identified a single point mutation (Val to Glu) at residue 664 in the transmembrane domain, which is responsible for cell transformation mediated by the mutated *HER-2/neu*.^{49,50} Subsequently, it was also found that substitution of Val with Gln at residue 664 created a mutated *HER-2/neu* with comparable transforming potential to that of Glu-664 mutant.⁵¹ Several studies have shown that point-mutated oncogenic p185 possesses higher tyrosine kinase activity than its normal counterpart.^{52,53} The increased tyrosine kinase activity provides point-mutated p185 with an increased autophosphorylation activity and an increased ability to transphosphorylate the tyrosine residues of its substrates,^{52,53} which are believed to be critical in oncogenic p185-mediated cell transformation. Although the mechanism of point-mutation activation of *HER-2/neu* is not very clear, recent studies have suggested that the increased tyrosine kinase activity of the oncogenic p185 is due to an increased tendency of this protein to form the homodimers, which are an activated form of the protein with an intrinsically higher tyrosine kinase activity.⁵⁴ Such a suggestion is compatible with the allosteric aggregation hypothesis,⁵⁵ which proposes that the ligand-stimulated dimerization of a receptor tyrosine kinase via extracellular domain interaction between 2 monomeric receptors results in the activation of the kinase activity. In a more recent report, Guy et al⁵⁶ compared the biochemical differences between the normal and transforming forms of insect cell-expressed *HER-2/neu* tyrosine kinases. The authors suggested the transmembrane domain of p185 may harbor a negative regulatory effect on the tyrosine kinase activity, and that switching the Val residue to Glu residue at position 664 in the transmembrane domain may relieve this constraint by altering the tertiary structure of the kinase active site, al-

lowing it to become more susceptible to activation on Mg²⁺ binding.

Overexpression in Cells

Interestingly, no analogous point mutation has been found yet in the human *HER-2/neu* gene product in tumors,⁵⁷⁻⁶⁰ although the human *HER-2/neu* can be activated by introducing the same codon substitution as that in oncogenic rat *HER-2/neu*.⁴⁸ One possible interpretation for this discrepancy is that double mutations (from Val [encoded by GTT] to Glu [encoded by GAA or GAG]) are required for generating the same amino acid change in the human gene, but only one point mutation (from Val [encoded by GTG] to Glu [encoded by GAG]) is sufficient to create the activation mutation in the rat *HER-2/neu*. In contrast to the cellular transformation mediated by the point mutation-activated rat *HER-2/neu*, the alteration occurring in human cancer cells is overexpression of a normal gene product that is frequently, but not always, due to gene amplification.^{16,57,61} In addition to the reported clinical data, experimental results also indicated that overexpression of normal human or rat *HER-2/neu* in NIH/3T3 cells was able to induce cellular transformation and tumorigenesis.⁹⁻¹¹ However, a critical level of overexpression is necessary to achieve transformation of NIH/3T3 cells,^{5,9,62} suggesting that normal *HER-2/neu* acts as a potent oncogene only when sufficiently overexpressed in NIH/3T3 cells.

The question remains as to how the oncogenic potential of normal *HER-2/neu* is activated under conditions of overexpression. To address this question, Greene's group expressed and purified p185 in insect cells via baculovirus expression vector.⁶³ By using a sucrose gradient, they were able to show that purified p185 existed mainly in the monomeric form at low concentrations, whereas at higher concentrations p185 existed as dimer or multimers.⁶³ The high molecular weight form of p185 is enzymatically more active. These results support the notion that con-

trol of the levels of p185 expression is critical to regulate the signaling ability of its constitutively active kinase. A disruption in the equilibrium caused by overexpression above a critical threshold might lead to progression of the cell along the neoplastic pathway.

Heterodimerization

In addition to the enhanced formation of the homodimer of p185 caused by point mutation and overexpression of *HER-2/neu*, heterodimer formation of EGFR and p185 has been shown to lead to the activation of p185 tyrosine kinases.^{62,64-67} This discovery was first made in the attempt by Stern and Kamps⁶⁴ to search for the ligand for p185. They screened known growth factors and tissue extracts for their ability to stimulate tyrosine phosphorylation of p185 in the rat embryonal fibroblast cell line (Rat-1). Surprisingly, these experiments revealed that incubation of Rat-1 cells with epidermal growth factor (EGF) stimulated tyrosine phosphorylation of p185 with a concomitant increase in its tyrosine kinase activity. This effect is specific to EGF because platelet-derived growth factor (PDGF), insulin, and transforming growth factor-beta do not induce tyrosine phosphorylation of p185.^{64,67} It was also found that EGF triggered a rapid tyrosine phosphorylation of p185 in the mammary tumor cell line, SK-BR-3,⁶⁵ and MKN-7 human adenocarcinoma cells.⁶⁶ It was subsequently demonstrated that the EGF-induced effect required interaction of the EGFR and EGF because cell lines lacking EGFR or with EGFR down-regulated by EGF could not phosphorylate p185, even when exposed to high concentrations of EGF.^{64,65,67} These results suggested that EGF, acting through EGFR, is able to regulate the intrinsic tyrosine kinase activity of p185, and that p185 is a substrate for EGFR. Direct experimental evidence for heterodimer formation came from cross-linking studies that indicated that p185 and EGFR associated into an active heterodimer, and that heterodimeriza-

tion was stimulated by exposure to EGF.⁶⁸ More recent data, however, indicated that p185 was not just a simple substrate for EGFR, but a transactivator as well.^{62,69,70} Co-expression of EGFR and p185 indicated that p185 could synergistically interact with EGFR, which led to transformation of NIH/3T3 cells.⁶² It has also been shown that co-expression of EGFR and kinase-deficient p185 abolished the synergistic transformation and tumorigenicity, and significantly suppressed the normal response of EGFR to ligand, although heterodimerization did occur and the mutated p185 was still transphosphorylated by associated EGFR.⁶⁹ Moreover, the heterodimer composed of wild-type EGFR and mutant p185 lacking a cytoplasmic domain is tyrosine kinase inactive, suggesting an inter-receptor activation mechanism.⁷⁰

Several groups have recently identified potential ligands for p185.⁷¹⁻⁷⁵ Two of them further reported purification, molecular cloning, and expression of a candidate ligand for p185.^{74,75} This ligand, called heregulin (HRG) or *neu* differentiation factor (NDF), stimulates tyrosine phosphorylation of p185 in some human mammary cancer cells. Subsequently, it was also found that the glial growth factors promoting the proliferation of cultured Schwann cells and the brain- or spinal cord-derived factor-inducing myotube differentiation (acetylcholine receptor-inducing activity) are identical to HRG/NDF.^{76,77} Interestingly, although HRG/NDF is able to stimulate the tyrosine phosphorylation of p185 in human tumor cells of breast, colon, and neuronal origin, it is incapable of acting on this receptor in certain ovarian cancer cells or *HER-2/neu*-transfected mouse fibroblast cells.⁷⁸ These observations have suggested that another member of the class I receptor subfamily is the receptor for HRG/NDF and that p185 becomes tyrosine-phosphorylated through a receptor heterodimerization mechanism, reminiscent of the EGFR/p185 inter-receptor activation.

More recently, it has been demonstrated that both p180^{HER-3/erbB-3} and

p180^{HER-4/erbB-4} are receptors for HRG/NDF, and the presence of one of these receptors is essential for tyrosine phosphorylation of p185 stimulated by HRG/NDF.⁷⁹⁻⁸¹ Plowman et al⁷⁹ expressed p185, p180^{HER-4/erbB-4}, or the 2 receptors together in a human T-lymphoblastoid cell line (CEM cell line) that does not express EGFR, p185, p180^{HER-3/erbB-3}, or p180^{HER-4/erbB-4}. They demonstrated that HRG/NDF was able to stimulate tyrosine phosphorylation of p180^{HER-4/erbB-4} in CEM cells expressing p180^{HER-4/neu} alone, but could only stimulate p185 tyrosine phosphorylation in cells expressing both receptors. In other studies, Carraway et al⁸⁰ employed the baculovirus insect cell expression system, in which insect cells do not express class I receptors, and the baculovirus life cycle prevents expressed foreign proteins from inducing the expression of endogenous receptors, to show the specific interaction of the HRG α and HRG β 1 isoforms with p180^{HER-3/erbB-3}. They also demonstrated that when expressed in mouse fibroblast cells, p180^{HER-3/erbB-3} mediated HRG/NDF-stimulated tyrosine phosphorylation of itself and endogenous p185. A simple and plausible model to interpret these observations would be that HRG/NDF binding to the ectodomain of either p180^{HER-3/erbB-3} or p180^{HER-4/erbB-4} induces the heterodimerization of that receptor with p185, stimulating the tyrosine kinase activity of the heterodimer. This model was tested using COS7 cells transiently expressing p185, p180^{HER-3/erbB-3}, or the 2 receptors together.⁸¹ Cells expressing p180^{HER-3/neu} alone possess a low-affinity HRG/NDF-binding site, with little or no stimulation of tyrosine phosphorylation in response to HRG β 1. In contrast, HRG β 1 binds to cells co-expressing p185 and p180^{HER-3/erbB-3} with a much higher affinity and induces tyrosine phosphorylation of both receptors. Direct interaction between HRG and the 2 receptors was demonstrated by chemical cross-linking experiments using iodinated HRG β 1 followed by immunoprecipitation with antibodies against p185 or p180^{HER-3/erbB-3}.⁸¹

The biologic significance of heterodimerization of p185 with other members of the class I receptor subfamily is not clear. One hypothesis is that inter-receptor activation and transphosphorylation of heterologous receptors may modulate growth regulatory signals by determining which signaling proteins containing *Src* homology domain 2 (SH2) are recruited to the activated receptor complex. The p185 homodimer may recruit a different subset of SH2-containing proteins and elicit different signals than a heterodimer of p185 with EGFR, p180^{HER-3/erbB-3}, or p180^{HER-4/erbB-4}.

Downstream Signaling Transducers of p185

Although aberrant tyrosine kinase activity of p185 has been shown to be absolutely linked to its transforming ability, and the activation mechanisms of p185 are relatively clear, the downstream signaling pathway of p185 is not well defined, mainly due to the absence of a consensus in regard to its ligand.⁷¹⁻⁸¹ Autophosphorylation of activated growth factor receptors and tyrosine phosphorylation of their substrates containing SH2 domains are believed to play an important role in intracellular signal transduction.⁸² Phospholipase C (PLC) γ and Ras-GTPase-activating protein (Ras-GAP) are 2 extensively studied substrates for p185⁸³⁻⁸⁵; and it has been shown that activated p185 is able to phosphorylate PLC γ and Ras-GAP on tyrosine residues and form stable complexes. Experiments with a chimeric EGFR-p185 receptor, consisting of the extracellular domain of EGFR and the transmembrane and intracellular domains of the p185, also demonstrated that tyrosine phosphorylation of PLC γ and Ras-GAP and association with EGFR-p185 chimeric receptor were ligand (EGF)-dependent.^{83,85} However, the importance of these 2 proteins in mediating the mitogenic signaling pathway of p185 is still inconclusive.^{83,84} Very recently, we compared the tyrosine phosphorylation levels of PLC γ and Ras-GAP in two NIH/3T3-derived

cell lines (transformed B104-1-1 and nontransformed dihydrofolate reductase (DHFR)/G8 cells), in which point-mutation activated and normal rat *neu* genes were transfected and expressed, respectively.⁸⁶ No significant difference could be detected for the total tyrosine phosphorylation levels of Ras-GAP and PLC γ between these 2 cell lines, suggesting that tyrosine phosphorylation of PLC γ and Ras-GAP does not correlate with the cellular transformation mediated by transforming *HER-2/neu*.

The Shc proteins have recently been described as SH2 domain-containing proteins that interact with various growth factor receptors, including the EGFR and p185.^{87,88} Overexpression of the *shc* gene induces transformation of NIH/3T3 cells.⁸⁷ The *shc* gene encodes 3 widely expressed proteins—p46^{shc}, p52^{shc}, and p66^{shc}. Interaction of *shc* with various activated receptor tyrosine kinases leads to tyrosine phosphorylation of the Shc proteins. Upon tyrosine phosphorylation, Shc proteins bind to the Grb2/ASH protein, an adaptor linking activated receptor tyrosine kinases to the Ras GTP-GDP exchange protein, Sos.⁸⁹ Thus, Shc may be involved in growth factor signaling to Ras and subsequent activation of mitogen-activated protein (MAP) kinase. The human p185 subdomain required for binding to Shc has been narrowed down to the very carboxyl-terminal 179 residues. Phosphorylation of one or more of the autophosphorylation sites within this p185 subdomain is required for Shc/p185-complex formation and is crucial for tyrosine phosphorylation of Shc.⁸⁸ Recently, we compared the tyrosine phosphorylation level of Shc in B104-1-1 and DHFR/G8 cells and found tyrosine phosphorylation of Shc was much more significant in B104-1-1 cells than in DHFR/G8 cells. Moreover, formation of the Shc/Grb2 complex was efficiently induced by point-mutation activated p185 in B104-1-1 cells, but hardly detected in DHFR/G8 cells.⁸⁶ Our results suggested that Shc may play an important role in the transformation of NIH/3T3 cells induced by activated p185. Compatible with

our observation, Ben-Levy et al⁹⁰ have recently demonstrated that the transforming ability of point mutation-activated *HER-2/neu* is mediated by Ras. This work was done by microinjection of a monoclonal anti-Ras antibody that can neutralize natural Ras proteins in murine fibroblasts. The authors found that injection of this anti-Ras into transformed NIH/3T3 cells, mediated by point mutation, activated rat *HER-2/neu*-induced phenotypic reversal in most cells. Furthermore, they showed that activation of MAP kinase and c-Jun correlated with the transforming potential of mutant p185. Based on our data and other reports, a potential pathway for activated p185 signaling (Figure 1) is that activation of p185 by point mutation or other mechanisms induces tyrosine phosphorylation of Shc and formation of the Shc/Grb2 complex, which passes the signal to Ras through Sos. Activated Ras, in turn, activates MAP kinase via Raf and MAP kinase kinase. Activated MAP kinase then enters the nucleus and phosphorylates transcriptional factors that turn on "p185-response" genes.

Another SH2 domain-containing protein, termed Grb7, has been found to be associated with p185 in certain breast cancer cell lines.⁹¹ Grb7, cloned by screening expression libraries with the tyrosine phosphorylated carboxyl terminus of EGFR as a probe, was mapped on mouse chromosome 11 to a region that also contains the *HER-2/neu* locus.⁹¹ Interestingly, it was found that Grb7 was amplified and overexpressed in accordance with *HER-2/neu* in a number of breast cancer cell lines, such as SKBR-3 and BT-474, and cancer tissues.⁹¹ Co-immunoprecipitation of Grb7 and p185 was observed in both SKBR-3 and BT-474 cells, indicating Grb7 binds tightly to p185. Association of Shc and Grb7 was also observed in EGF-stimulated SKBR-3 cells.⁹¹ Sequence comparison indicated that Grb7 contained a pleckstrin domain, first described by Mayer et al,⁹² as well as Haslam and co-workers,⁹³ which is found in several different signaling proteins, including pleckstrin,⁹⁴ akt/ τ ac serine

kinase,^{95,96} Ras-GAP,^{97,98} and 3BP-2 SH3 binding protein.⁹⁹ It has been suggested that pleckstrin motif may be a site for protein-protein interaction.^{92,93} Although it remains to be determined which signal is relayed by Grb7, it is conceivable that Grb7 possesses a basic signaling function in the signaling pathway of activated p185.

Many primary human breast cancers have recently been reported to show elevated activities of pp60c-src (a nonreceptor tyrosine kinase), as well as p185.¹⁰⁰ Interestingly, the GST-Src fusion protein is able to precipitate phosphorylated p185, as well as EGFR from SKBR-3 breast cancer cells.¹⁰¹ These preliminary results raise a possibility that association of nonreceptor tyrosine kinase with p185 may be an integral part of the signaling pathway elicited from the activated p185.

Although autophosphorylation of p185 is commonly thought to play an important role in the mitogenic pathway of p185, and a number of SH2 domain-containing potential downstream transducer proteins have been described, no direct identification of individual autophosphorylation sites with which SH2 domain-containing ligands interact has been reported, probably due to the proximity and overlapping binding of several of the autophosphorylation sites. All potential autophosphorylation sites of p185 are clustered in the carboxyl-terminal tail.^{102,103} Recently, a novel peptide binding and selection technique has been employed by Songyang et al^{104,105} to predict potential docking sites in receptor tyrosine kinases and other signaling proteins for interaction with specific SH2 domain-containing proteins on the basis of the amino acid sequences surrounding the phosphorytyrosine residue. A list of possible autophosphorylation sites of p185 and other class I receptors and the SH2 domain-containing proteins with which they might interact was given in a recent review.¹⁰⁶ Recent studies suggested that individual SH2 domain-containing proteins may bind more than one autophosphorylation site of EGFR, and that

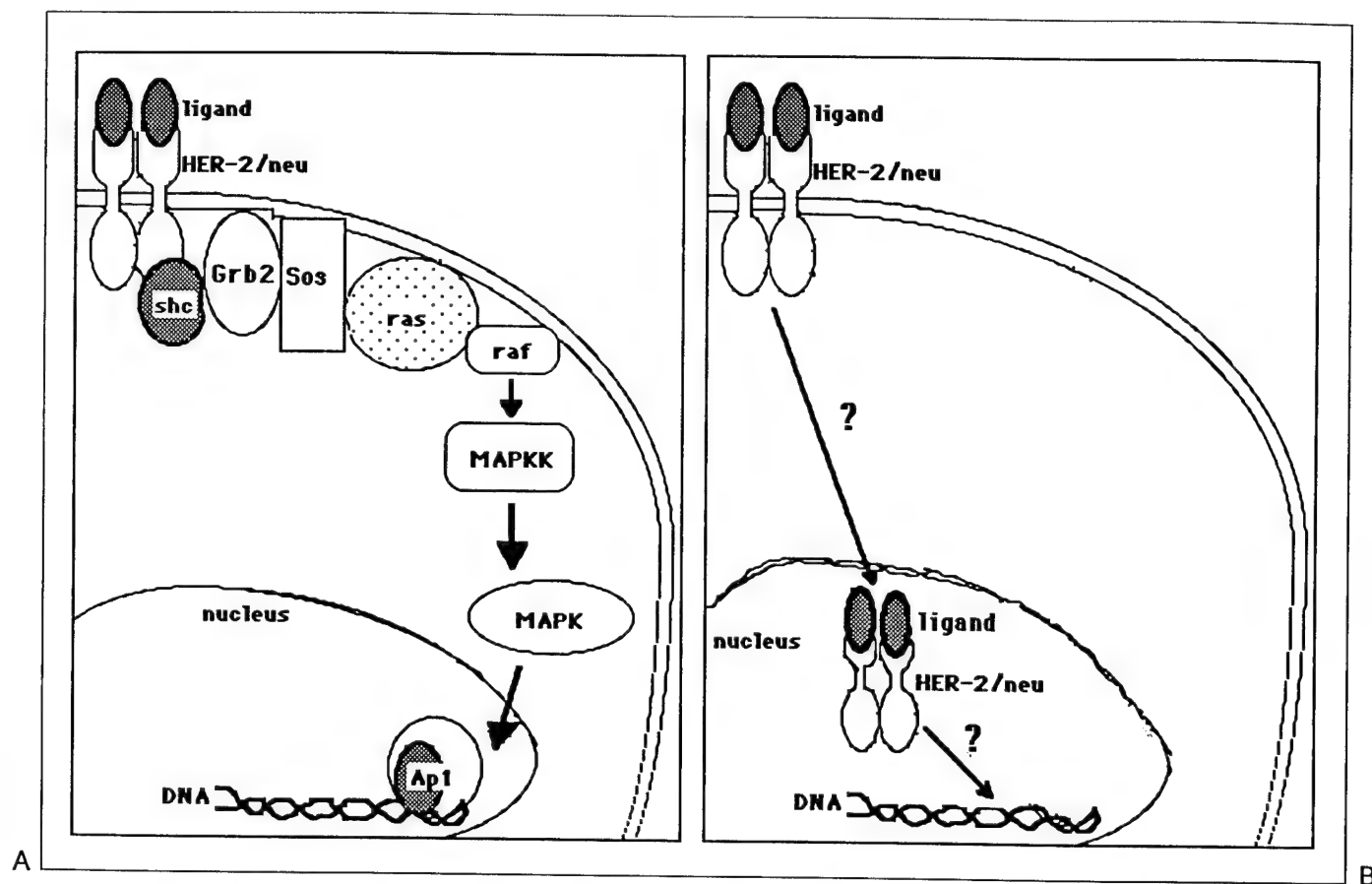


Figure 1. Hypothetic diagram of the signal transduction pathways of HER-2/neu. **(A)** Ligand binding activates HER-2/neu, causing it to dimerize and complex with downstream substrates. Activated substrates initiate a cascade reaction as shown in the diagram, which ultimately leads to the activated MAPK to translocate to the nucleus and phosphorylate Ap1 transcription factors so as to activate transcription of genes regulated by HER-2/neu. **MAPKK** indicates mitogen-activated protein kinase kinase; **MAPK**, mitogen-activated protein kinase. **(B)** Ligand binding leads to translocation of a small fraction of the receptor-ligand complex into the nucleus by some unknown mechanism. The receptor-ligand complex may influence transcriptional activity of specific genes.

one autophosphorylation site may interact with 2 different SH2 domain-containing proteins.¹⁰⁷ For example, Y-1068 and Y-1086 of EGFR are the primary and secondary binding sites, respectively, for Grb2. In addition, the major and secondary binding sites for Shc are Y-1173 and Y-992, respectively. On the other hand, Y-992 is the potential docking site for both PLC γ and Shc.^{107,108} This hierarchy of interaction may be caused by the high local concentration of autophosphorylation sites in the class I receptor subfamily.

A recent report demonstrated that, on stimulation with EGF, an EGFR mutant lacking all the autophosphorylation sites can still induce tyrosine phosphorylation of Shc, Shc/Grb2 complex formation, and mediate mitogenesis.¹⁰⁹ Our

own data also indicated that a deletion mutant of the point mutation-activated p185 that is incapable of autophosphorylation was able to induce tyrosine phosphorylation of Shc, formation of the Shc/Grb2 complex, and mediate transformation of NIH/3T3 cells.⁸⁶ Taken together, these data suggest that a similar SH2 domain repertoire could be recruited to the mutant receptors by a parallel pathway that does not involve direct interactions of SH2 domain-containing proteins and the mutant receptors.

A Novel Short-Cut Signaling Pathway of p185?

Recently, we used a 2-hybrid selection system to search for the signaling transducers of p185 and found,

surprisingly, that the cytoplasmic domain of p185, when fused to the DNA-binding domain of GAL4 (amino acids 1-147), functioned as a transcriptional activator.¹¹⁰ We subsequently observed nuclear localization of p185. Interestingly, nuclear p185 has a much higher extent of tyrosine phosphorylation than its non-nuclear counterpart. Our results suggest that a transmembrane receptor tyrosine kinase may enter the nucleus and be involved in transcriptional activation.

It is commonly thought that most receptor growth factor complexes are internalized, transported to lysosomes, and degraded after ligand binding and receptor activation. Recently, however, experiments demonstrated that a number of growth factors could directly exert their effect in the nucleus.

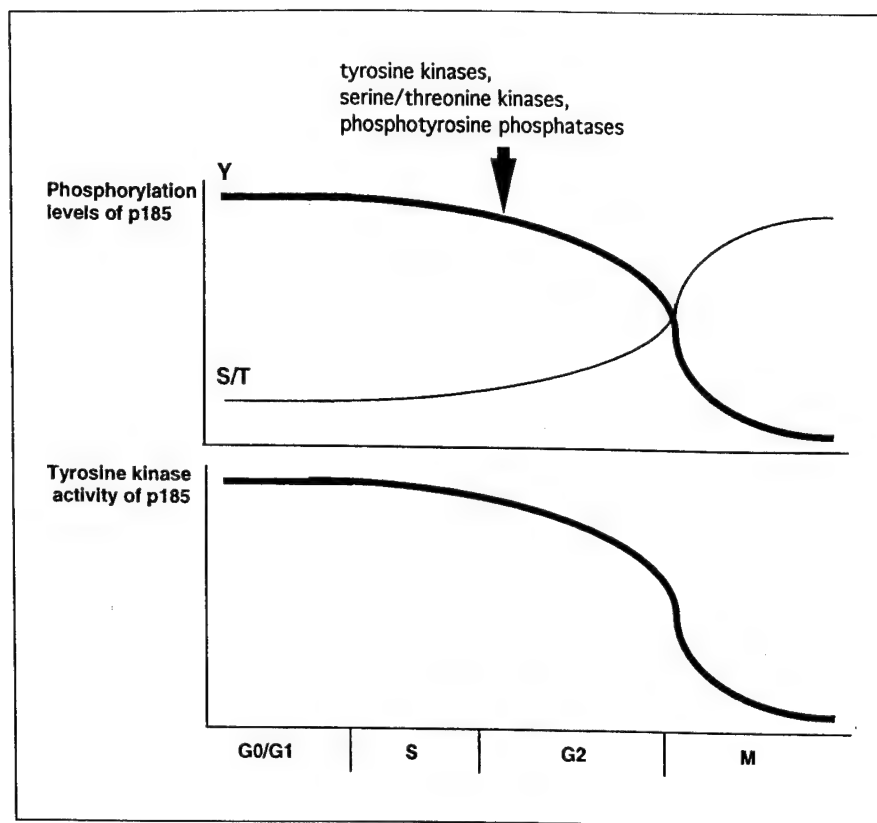


Figure 2. Diagram shows cell cycle-dependent regulation of p185 tyrosine kinase. The phosphotyrosine content of p185 is at its highest level in G0/G1 phase, decreases through S and G2 phases, and reaches its lowest level in M phase. In contrast, serine and threonine residues are hyperphosphorylated in M phase. These changes on phosphorylation are revealed as the functional change of p185 tyrosine kinase, namely p185 is most active in G0/G1 phases and is least active in M phase in tyrosine phosphorylation function. A series of serine/threonine kinases such as cdc2 and phosphotyrosine phosphatases are tightly regulating the function and phosphorylation of p185. Y indicates tyrosine; S, serine; and T, threonine.

For example, basic fibroblast growth factor (bFGF) accumulates in the nucleus, and this translocation to the nucleus is cell-cycle specific (in late G1), consistent with the possibility that bFGF could play a role in replication and/or transcription of rDNA.¹¹¹ Subsequently, bFGF has been shown to regulate gene transcription in a cell-free system.¹¹² Epidermal growth factor, nerve growth factor (NGF), and PDGF have also been found to accumulate in the nucleus of cells bearing their respective receptors.¹¹³ Moreover, it has been observed that Schwannoma-derived growth factor (SDGF), a ligand for EGF receptor, binds to A+T-rich DNA sequences, and transport into the nucleus is required for SDGF to induce a mitogenic response.¹¹⁴ Compatible with

these demonstrations showing the possibility that growth factors are translocated to the nucleus where they exert their effect, our observation of nuclear localization of p185, and its association with a potential transcriptional activation activity, may unveil a novel pathway for the signal transduction of the tyrosine kinase receptors. One attractive model (Figure 1), yet to be tested, would be that growth factors might bind to specific DNA sequences, and growth factor receptors interacting with respective growth factors could trigger transactivation function. Because the ligand for p185 is not yet well defined, it is difficult to investigate how p185 is translocated into the nucleus. We did, however, observe that EGFR could be translocated to the nucleus on binding to

EGF by using chemical cross-linking approaches (Matin and Hung, unpublished observation, 1994). These studies may provide a new avenue for understanding the signal transduction pathway of receptor tyrosine kinases.

A New View on the HER-2/neu-Induced Transformation

In studying cell-cycle regulation of rat p185 phosphorylation and tyrosine kinase activity, we found that tyrosine phosphorylation of p185 was predominant in G0/G1 phase, decreased through S and G2 phases, and reached its lowest level in M phase (Figure 2).¹¹⁵ Compatible with these observations, we demonstrated that the fluctuation profile of the tyrosine kinase activity of p185 through the cell cycle was identical to that of the change of phosphotyrosine content in p185 (Figure 2).¹¹⁵ In contrast to tyrosine phosphorylation, p185 was hyperphosphorylated on Ser and/or Thr residues in M phase. Furthermore, we observed that cdc2 kinase was able to phosphorylate p185 most efficiently in M phase, when the autophosphorylation activity of p185 was inactivated.¹¹⁵ Taken together, these data suggest the structure and function of p185 are under a stringent cell cycle-dependent regulation. However, the point mutation-activated rat p185 escapes this cell cycle regulation and constitutively exerts its tyrosine kinase activity throughout the cell cycle. These results suggest that a disruption of cell cycle-regulated tyrosine kinase activities may play a role in the cellular transformation mediated by the point mutation-activated rat p185.

Conclusion

Overexpression and/or amplification of *HER-2/neu* is frequently detected in many human cancers. Activation of p185 tyrosine kinase can be achieved by point mutation, overexpression, and heterodimerization with other class I receptors. The tyrosine kinase activity of normal p185 is cell-cycle dependent; however, the

tyrosine kinase activity of the point-mutation-activated rat p185 is cell-cycle independent, which may lead to cellular transformation. Studies of the signal transduction of p185 indicate that a series of SH2 domain-containing intermediate proteins are involved in the signaling of activated p185 through protein-protein interactions. A conceivable pathway is activated p185-Shc-Grb2-Sos-Raf-MAPKK-MAPK-AP1-target genes. Alternative pathways may also exist. For example, after binding its ligand, p185 may be translocated to the nucleus, where it exerts its transcriptional transactivation function. Further investigation of the signal transduction pathways of p185 will be beneficial in designing efficient approaches for *HER-2/neu* targeting gene therapy.

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